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Enhanced activity of recombinant β-secretase from *Drosophila melanogaster* S2 cells transformed with cDNAs encoding human β1,4-galactosyltransferase and Galβ1,4-GlcNAc α2,6-sialyltransferase

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Abstract

β-Secretase (βSEC) was expressed in *Drososphila melanogaster* Schneider 2 (S2) cells transformed with cDNAs encoding β1,4-galactosyltransferase (GalT) and Galβ1,4-GlcNAc α2,6-sialyltransferase (ST). The apparent molecular weight of recombinant β-secretase was increased from 56 kDa to 61 kDa. A lectin blot analysis indicated that recombinant β-secretase from S2βSEC/GalT-ST cells (S2 cells co-transformed with cDNAs encoding β-secretase, glycosyltransferases, GalT, and ST) contained the glycan residues of β1,4-linked galactose and α2,6-linked sialic acid. Two dimensional electrophoresis revealed that recombinant β-secretase from S2βSEC/GalT-ST cells had a lower isoelectric point compared to β-secretase from control S2βSEC cells (S2 cells transformed only with β-secretase cDNA). Recombinant β-secretase from transformed S2 cells was also present as heterogeneous forms. The enzyme activity of recombinant β-secretase from S2βSEC/GalT-ST cells was enhanced up to 260% compared to control S2βSEC cells. We have shown that an exogeneous human glycosyltransferases cDNA can be introduced into S2 cells to extend the N-glycan processing capabilities of the insect cell line, and that the extended glycosylation improves the activity of recombinant β-secretase.

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Keywords: Recombinant β-secretase; *Drosophila melanogaster* S2 cells; Expression; Activity; β1,4-Galactosyltransferase; Galβ1,4-GlcNAc α2,6-sialyltransferase

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1. Introduction

Alzheimer's disease is a neurodegenerative disorder that is characterized by neuronal loss in the brain and

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by the presence of amyloid plaques and neurofibrillary tangles (Selkoe, 1991). The major protein component of the plaques is amyloid β-peptides (Aβ) (Glenner and Wong, 1984). Formation of Aβ requires the proteolytic activities of both β-secretase (βSEC) and γ-secretase (Haass et al., 1994) for proteolytic cleavage of the β-amyloid precursor protein (Kang et al., 1987), which is a type-I transmembrane protein that is constitutively expressed in many cell types.

β-Secretase is one of the prime targets for therapeutic intervention in Alzheimer's disease. A steady supply of large quantities of an active recombinant β-secretase is a prerequisite for development of a secretase inhibitor. In this respect, recombinant β-secretase has been expressed in insect cells using baculovirus-insect cell systems (Charlwood et al., 2001; Bruinzeel et al., 2002) and *Drosophila melanogaster* Schneider 2 (S2) expression systems (Mallender et al., 2001). A high mannose glycosylated β-secretase reduced enzyme activity up to 50% in baculovirus-infected insect cells, and unglycosylated β-secretase from tunicamycin-treated recombinant CHO cells exhibited 40% of the activity of the glycosylated β-secretase from untreated recombinant CHO cells (Charlwood et al., 2001). Thus, the glycosylation state of recombinant β-secretase apparently is important in maintaining enzyme activity. However, recombinant expression of soluble β-secretase in insect cell systems can result in limiting N-glycan processing of glycoproteins due to a deficiency in the glycosyltransferases necessary for production of the complex oligosaccharide.

In a recent study, baculovirus expression of both human transferrin and β1,4-galactosyltransferase (GalT) in high five cells yielded additional galactose-containing hybrid oligosaccharide (Ailor et al., 2000). Baculoviral expression of both β1,4-galactosyltransferase and α2,6-sialyltransferase (ST) along with the baculoviral envelope protein gp64 in *Spodoptera frugiperda* 9 and high five cells was sufficient to obtain sialylglycans (Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001). Baculoviral expression of three glycosyltransferases, GlcNAc transferase II, β1,4-galactosyltransferase, and α2,6-sialyltransferase in Ea4 cells yielded sialylated α1-antitrypsin glycoproteins (Chang et al., 2003). These findings indicate that extension of the N-gylcan structure by expression of the glycosyltransferases GalT and ST in transformed *D. melanogaster* S2 insect cells is feasible, and that extended glycosylation can improve the activity of recombinant β-secretase. Therefore, we have characterized recombinant β-secretase from *D. melanogaster* S2 cells transformed with cDNAs encoding human β1,4-galactosyltransferase and Galβ1,4-GlcNAc α2,6-sialyltransferase.

2. Materials and methods

2.1. Cell line, plasmids, and enzymes

D. melanogaster Schneider 2 cells were grown at 27 °C in T-25 (Nunc, Denmark) flasks in Shields and Sang M3 insect medium (Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) with a 10% insect medium supplement (IMS; Sigma). The plasmid pMT/BiP/V5-His (3.6 kb; Invitrogen, Carlsbad, CA, USA) has a metallothionein promoter and a BiP signal sequence. The selection plasmid pCoHygro (Invitrogen), the bacterial hygromycin B phosphotransferase gene under control of the constitutive *Drosophila* Copia 5′LTR promoter, was also used. *E. coli* JM109 was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3) containing 50 μg ml^{-1} of ampicillin with agitation at 37 °C. DNA restriction enzymes from either Promega (Madison, WI, USA) or Takara (Otsu, Shiga, Japan) were used according to the manufacturer's instructions.

2.2. Construction of expression plasmids

The human β-secretase gene was amplified from plasmid DNA, pcDNA3.1-βSEC by PCR with sense (5′-AGATCTACCCAGCACGGCATC-3′) and antisense (5′-CTCGAGATAGGCTATGGTCATG-3′) primers. The amplified 1317-base pair fragment was inserted into the pGEM-T vector to yield pGEM-T-βSEC. pMT/BiP-βSEC-V5-His (Fig. 1A) was constructed by inserting *Bgl*II and *Xho*I fragments of pGEM-T-βSEC between the *Bgl*II and *Xho*I sites of pGEM-T-βSEC. The cDNAs encoding human β1,4-galactosyltransferase and Galβ1,4-GlcNAc α2,6-sialyltransferase were synthesized by RT-PCR using total RNA isolated from human placenta cells (Kim

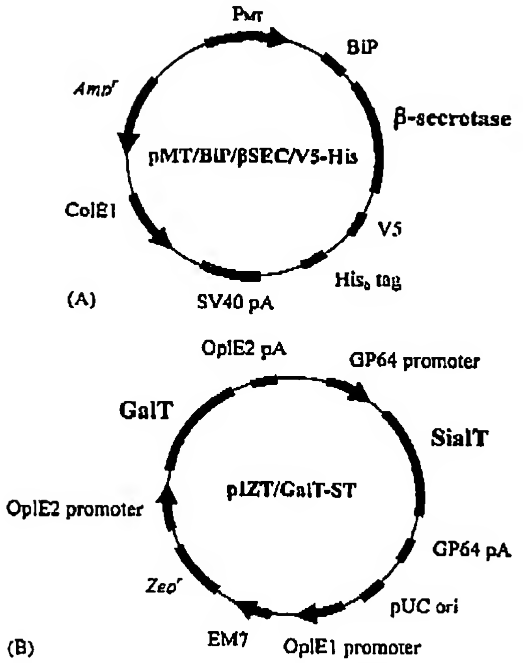

Fig. 1. A schematic representation of the expression plasmids. (A) pMT/BiP/βSEC/V5-His; (B) pIZT/GalT-ST.

et al., 2003). cDNAs were subcloned into pIZT for constitutive expression in S2 cells. pIZT/GalT-ST was designed to express human β1,4-galactosyltransferase (hGalT I, EC2.4.1.22) and Galβ1,4-GlcNAc α2,6-sialyltransferase (hST6Gal I, EC 2.4.99.1) in S2 cells (Fig. 1B). The proper orientation and the reading frame of the inserted gene in all recombinant plasmids were confirmed by both restriction enzyme mapping and DNA sequencing.

2.3. Stable transformation, cell culture, and analysis of gene expression

S2 cells were co-transfected with pMT/BiP-βSEC-V5-His, pIZT/GalT-ST, and pCoHygro using the lipofectin method (Park et al., 2001). S2 cells as a control were co-transfected with pMT/BiP-βSEC-V5-His and pCoHygro. Stably transformed polyclonal cell populations of S2 cells were isolated after 4 weeks of selection with both hygromycin and zeocin, or only with hygromycin. S2βSEC/GalT-ST cells (S2 cells co-transformed with cDNAs encoding β-secretase and glycosyltransferases, GalT, and ST) were grown at 27 °C in T-25 flasks in 3 ml of M3 medium containing 10% IMS, 300 μg ml^{-1} of hygromycin B, and 100 μg ml^{-1} of zeocin. S2βSEC cells as a control (S2 cells transformed with only β-secretase cDNA) were grown at 27 °C in T-25 flasks in 3 ml of M3 medium containing 10% IMS and 300 μg ml^{-1} of hygromycin B. Unless otherwise specified, stably transformed S2 cells were cultured for 7 days to analyze recombinant protein expression. Stably transformed S2 cells were induced with 0.5 mM CuSO$_4$ after the start of the run. Cultures of S2 cells were centrifuged at 3000 rpm for 5 min to separate the cells. The supernatant was used to identify extracellular recombinant proteins. The cell fraction was rocked for 1 h in lysis buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg ml^{-1} of phenylmethylsulfonyl fluoride, 1 μg ml^{-1} of aprotinin, and 1% Triton X-100) and subjected to three freeze-thaw cycles of 10 min in a −70 °C freezer and 5 min in a 37 °C water bath. After centrifuging cell extracts at 14,000 rpm for 15 min to remove cell debris, the supernatant was used to identify intracellular recombinant proteins.

2.4. Southern blot analysis

Genomic DNA was prepared from both S2βSEC and S2βSEC/GalT-ST cells using a standard procedure (Sambrook et al., 1989), and 10 μg aliquots were digested with either *Eco*RI and *Pst*I or *Eco*RI and *Kpn*I. A probe was prepared from a 600-bp *Eco*RI and *Pst*I fragment of pIZT/GalT-ST, including part of the coding region of human β1,4-galactosyltransferase, and also from a 680-bp *Eco*RI and *Kpn*I fragment of pIZT/GalT-ST, including part of the coding region of human Galβ1,4-GlcNAc α2,6-sialyltransferase. The digests and equivalents were resolved on 0.8% agarose gel and transferred to Hybond-N membranes following the manufacturer's instructions (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Membranes were prehybridized for 2 h at 65 °C in 6× SSC (1× is 0.15 M NaCl/15 mM sodium citrate, pH7)/5× Denhardt's solution/0.5% SDS with 0.1 mg ml^{-1} of denatured salmon sperm DNA was hybridized for an additional 16 h in the same solution with a ^{32}P-labeled probe labeled by Prime-a-Gene Labeling System (Promega). After hybridization, membranes were washed in 2× SSC/0.1% SDS at 65 °C for 10 min, in 1× SSC/0.1%

SDS at 65 °C for 10 min, and finally in 0.5× SSC/0.1% SDS at 65 °C for 10 min, then sealed in a bag. Kodak (Rochester, NY, USA) X-ray film was then exposed to the membranes. The band intensity of the blot was quantified by densitometry. The copy number of a target genomic DNA sequence was estimated by measuring the intensity of hybridization signals of genomic bands and comparing the intensity of signals by bands that contain known gene equivalents of the target sequence. The equivalents of 1, 5 and 10 copies of the target sequence relative to 1 µg of *Drosophila* S2 cell genome DNAs were calculated using the equation (Pasternak, 1993), $X = g/G \times v/i$, where G is the genome size in bp, g the size of the target gene in bp, i the size of the insert in bp in the cloning vector, and v is the size of the vector plus the insert in bp. X gives the quantity in µg of a "vector with insert" DNA sample that is equivalent to one copy per genome of the target sequence.

2.5. SDS-PAGE and Western blot analysis

Protein samples that were separated by electrophoresis on 8–10% polyacrylamide-SDS gel (Laemmli, 1970) were visualized by silver staining (Sambrook et al., 1989) and subjected to Western blot analysis. The electrophoresed proteins on the gel were transferred to a nitrocellulose membrane (Amersham-Pharmacia Biotech, Piscataway, NJ, USA), blocked with 3% skim milk, incubated with mouse anti-V5, (1:1000 dilution in TBS, Invitrogen), and probed with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:1000 dilution in TBS, Sigma). The membranes were washed and BCIP/NBT solution (Amresco, Solon, OH, USA) was added. The reaction was quenched with distilled water.

2.6. Purification of recombinant β-secretases

S2βSEC/GalT-ST cells were cultured for 7 days in T-75 flasks with 8 ml of M3 medium containing 10% IMS, 0.5 mM $CuSO_4$, 300 µg ml^{-1} of hygromycin B, and 100 µg ml^{-1} of zeocin. S2βSEC cells were cultured 7 days in T-75 flasks with 8 ml of the M3 medium described above with no zeocin. These stably transformed S2 cells were centrifuged at 3000 rpm for 5 min to harvest the medium, which was then dialyzed in Tris buffer (20 mM Tris–Cl, 500 mM NaCl, pH 7.9) for 48 h. The dialyzed medium was added to imidazole to a final concentration of 10 mM and incubated with fast-flow Ni-NTA resin (Qiagen, Valencia, CA, USA) in a chromatography column (Novagen, Madison, WI, USA), according to the manufacturer's instructions. Weakly bound proteins were washed with three volumes of Tris buffer containing 10 mM imidazole. Recombinant β-secretases from S2βSEC and S2βSEC/GalT-ST cells were then eluted with Tris buffer containing 200 mM imidazole. Fractions containing the recombinant protein were pooled and dialyzed in buffer containing 20 mM Tris–Cl (pH 6.0) to remove the imidazole.

2.7. Lectin blot analysis of recombinant β-secretases

After quantification by Western blotting, equivalent amounts of purified recombinant β-secretases were used for lectin blotting assays (Jarvis and Finn, 1996). Membranes were cut into strips corresponding to individual lanes, blocked, and probed with either mouse anti-V5 or biotinylated (Vector Laboratories, Burlingame, CA, USA) lectins. The lectins used in this study were *Ricinus communis* agglutinin (RCA), which binds β-linked galactose, and *Sambucus nigra* agglutinin (SNA), which binds terminal α2,6-linked sialic acid. Bound lectins and antibodies were detected by secondary reactions with alkaline phosphatase-conjugated goat anti-mouse IgG antibody and alkaline phosphatase-conjugated avidin (Vector Laboratories, Burlingame, CA, USA). The membranes were washed and BCIP/NBT solution (Amresco, Solon, OH, USA) was added. For lectin blotting using RCA, purified β-secretase was pretreated with 2000 U ml^{-1} of *Arthobacter urefaciens* neuraminidase (Calbiochem, LaJolla, CA, USA) for 6 h at 37 °C (Laemmli, 1970). After the incubation period, pretreated β-secretases were analyzed by SDS-PAGE and lectin blotting.

2.8. Two-dimensional electrophoresis

For the first-dimension, isoelectric focusing was performed with commercially available immobilized pH gradient (IPG), pH 4–7 gels (Immobiline Drystrip gel, pH 4–7, 13 cm length, Amersham-Pharmacia Biotech, Piscataway, NJ, USA) and an IPGphor

Isoelectric Focusing System (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Purified recombinant protein samples were dialyzed in Tris buffer (20 mM Tris–Cl, pH 7.9) for 12 h, then an amount of 8 volumes of sample buffer was added (10 M urea, 2.4% CHAPS), along with 0.5% IPG buffer and 2.5% 2-ME. The mixture was incubated at room temperature for 10 min, then loaded onto IPG gel. Isoelectric focusing was performed at 100 V for 1 h, 500 V for 3 h, and 1500 V for 7 h at 20 °C. After first dimensional isoelectric focusing, the strip gel was equilibrated in a buffer (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). The strip was then placed on top of the slab gel for SDS-PAGE in the second dimension.

2.9. β-Secretase activity assay

Protein concentrations were measured by the Bradford method using an RC DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. To examine the enzymatic activities of purified recombinant β-secretases from S2βSEC and S2βSEC/GalT-ST cells, reactions were performed in a buffer of 0.1 M sodium acetate, pH 4.5, at 37 °C with 10% dimethylsulfoxide (DMSO) and a 20 μM β-secretase substrate, H-RE(EDANS)EVNLDAEFK(DABCYL)R-OH (FS-1; Calbiochem: LaJolla, CA, USA). A change in the fluorescence intensity during substrate hydrolysis was measured using a fluorometer (Turner Digital Fluorometer, Model 450, Barnstead/Thermolyne, USA). An excitation wavelength of 360 nm and an emission wavelength of 515 nm were used to monitor hydrolysis of the β-secretase substrate. In a kinetic study to determine the Michaelis constant (K_m) of the substrate for purified recombinant β-secretase, reactions were performed in a buffer of 0.1 M sodium acetate, pH 4.5, at 37 °C with 10% dimethylsulfoxide at substrate concentrations of 1, 1.5, 5, 10, 15, and 20 μM. Inhibition studies were performed at a substrate concentration of 20 μM with different concentrations of the inhibitor, (Asn^{670}, Sta^{671}, Val^{672})-Amyloid β/A4 Protein Precursor$_{770}$ (662–675) (Bachem, LaJolla, CA, USA) in the reaction mixture. The Michaelis constant and the concentration at half-maximum inhibition (IC_{50}) were obtained from a fit of the data using GraphPad Software (GraphPad PRISM, Version 4.0) (Heo et al., 2001).

3. Results and discussion

3.1. Analysis of genomic DNAs in S2βSEC and S2βSEC/GalT-ST cells

The N-linked glycans from most insect cell-derived glycoproteins lack a penultimate galactose and a terminal sialic acid (Marz et al., 1995; Jarvis and Finn, 1995; Tomiya et al., 2003). One of the reasons is that insect cells generally appear to have an extremely low level of galactosyltransferase activity and no detectable sialyltransferase activity to convert most N-linked side chains to complex forms (Hollister and Jarvis, 2001). We have attempted to extend the glycosylation of recombinant β-secretase expressed in the *D. melanogaster* S2 (S2) cell line by introducing human glycosyltransferase genes (β1,4-galactosyltransferase and Galβ1,4-GlcNAc α2,6-sialyltransferase) into the cell line. Stably transformed S2 cells co-transfected with cDNAs encoding β-secretase and glycosyltransferases were obtained and designated as S2βSEC/GalT-ST cells. Stably transformed S2 cells transfected with only β-secretase cDNA were also obtained as a control and designated as S2βSEC cells. Southern blot analysis was used to investigate the insertion of glycosyltransferases genes in stably transformed S2 cells (Fig. 2). Ten microgram of genomic DNAs extracted from S2βSEC and S2βSEC/GalT-ST cells was digested with restriction enzymes and the digests were examined by Southern blot analysis. For a control, 1, 5, and 10 genome equivalents of pIZT/GalT-ST relative to 10 μg of *Drosophila* S2 cell genome DNAs were also subjected to Southern blot analysis (lanes 3, 4, 5 in Fig. 2). Two probes (prepared from a 600-bp *Eco*RI and *Pst*I fragment, and from a 680-bp *Eco*RI and *Kpn*I fragment of pIZT/GalT-ST) did not hybridize with genomic DNA from S2βSEC cells (lane 1 in Fig. 2), but did hybridize with genomic DNA from S2βSEC/GalT-ST cells (lane 2 in Fig. 2). Fig. 2A (Southern blot analysis using the probe prepared from the 600-bp *Eco*RI and *Pst*I fragment) shows the blot intensity for genomic DNAs from S2βSEC/GalT-ST cells (lane 2) with an intensity similar to five genome equivalents (equals to five copies per genome of the target sequence) (lane 4) based on densitometry. Fig. 2B (Southern blot analysis using the probe prepared from the 680-bp *Eco*RI and *Kpn*I fragment) shows the blot intensity for genomic

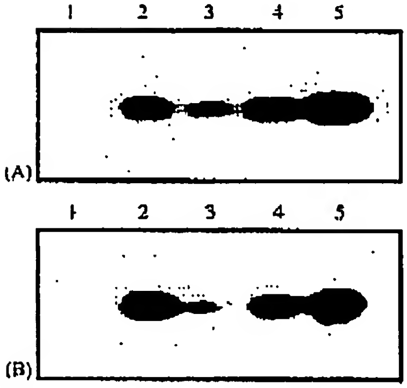

Fig. 2. Southern blot analysis of genomic DNAs from S2 cells. Ten microgram of genomic DNAs extracted from S2βSEC and S2βSEC/GalT-ST cells were digested with restriction enzymes. All digests were resolved by agarose gel electrophoresis as follows: lane 1 for (A) and (B), S2βSEC (S2 cells transformed with only β-secretase cDNA) cells; lane 2 for (A) and (B), S2βSEC/GalT-ST (S2 cells transformed with cDNAs coding β-secretase and glycosyltransferases) cells. The probe for (A) was a 600 bp fragment of human β1,4-galactosyltransferase cDNA. The probe for (B) was a 680 bp fragment of human Galβ1,4-GlcNAc α2,6-sialyltransferas cDNA. Lanes 3, 4, and 5 for A and B represent 1, 5, and 10 genome equivalents of pIZT/GalT-ST relative to 10 μg of *Drosophila* S2 cell genome DNAs.

DNAs from S2βSEC/GalT-ST cells (lane 2) with an intensity between 5 and 10 genome equivalents (lanes 3 and 5) based on densitometry. This analysis indicates that multiple copies of the glycosyltransferases genes were integrated into the S2 cell genome.

3.2. Expression of recombinant β-secretase in S2βSEC and S2βSEC/GalT-ST cells

The recombinant β-secretase expressed in control S2βSEC cells and confirmed by Western blot analysis (Fig. 3A) was present primarily in the medium fraction. The molecular weight (MW) was approximately 56 kDa, which is similar to the MW (56–57 kDa) reported for recombinant β-secretase in the S2 expression system (Mallender et al., 2001). β-Secretase secreted into the medium fraction of S2βSEC and S2βSEC/GalT-ST cell cultures was examined by Western blot analysis (Fig. 3B). The molecular weight of the secreted recombinant β-secretase expressed in S2βSEC/GalT-ST cells was approximately 61 kDa. The difference in the apparent molecular weight of secreted recombinant β-secretase from transformed S2 cells was approximately 5 kDa. The molecular size of the recombinant β-secretase from S2βSEC/GalT-ST cells was probably increased by functional expression of GalT and ST in transformed S2 cells.

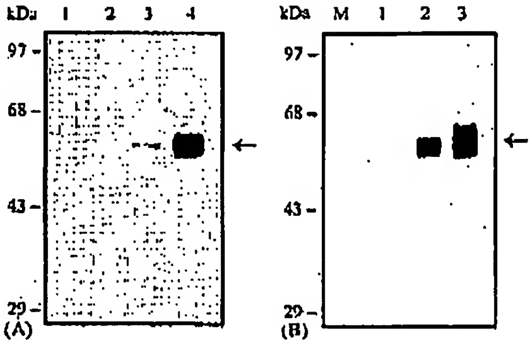

Fig. 3. Western blot analysis of S2 cells. The numbers on the left and the arrow indicate the molecular weight markers (kDa) and the recombinant β-secretase protein, respectively. (A) Western blot analysis of non-transfected S2 and S2βSEC (S2 cells transformed with only β-secretase cDNA) cells. (1) Cellular fraction of non-transfected S2 cells; (2) medium fraction of non-transfected S2 cells; (3) cellular fraction of S2βSEC cells; (4) medium fraction of S2βSEC cells. (B) Western blot analysis of S2βSEC/GalT-ST (S2 cells transformed with cDNAs encoding β-secretase and glycosyltransferases), and control S2βSEC cells (S2 cells transformed with only β-secretase cDNA). (1) Medium fraction of non-transfected S2 cells; (2) medium fraction of S2βSEC cells; (3) medium fraction of S2βSEC/GalT-ST cells.

3.3. Lectin blot analysis of recombinant β-secretase from S2βSEC and S2βSEC/GalT-ST cells

Transformed S2 cells were analyzed for production of recombinant β-secretases containing a β1,4-linked galactose and an α2,6-linked sialic acid (Fig. 4). The recombinant β-secretases tested were purified from S2βSEC and S2βSEC/GalT-ST cells by Ni^{2+}-affinity purification. Purified β-secretases were obtained from cultures of S2βSEC and S2βSEC/GalT-ST cells without visible contaminating proteins on silver nitrate-stained SDS-PAGE gel (data not shown). Lectin blot analysis revealed that *R. communis* agglutinin, which is specific to β-linked galactose, bound only to β-secretases produced by S2βSEC/GalT-ST cells (lane 2B, Fig. 4). *S. nigra* agglutinin, which specifically rec-

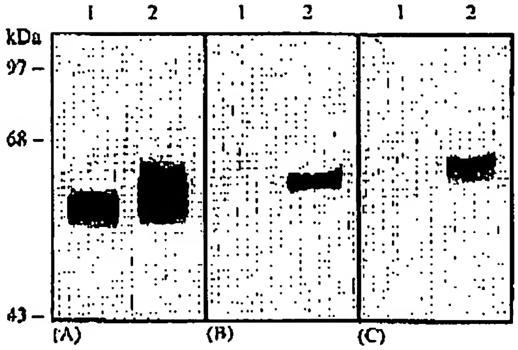

Fig. 4. Glycosylation of recombinant β-secretase from transformed insect cell lines. Recombinant β-secretases from S2βSEC (lane 1), and S2βSEC/GalT-ST (lane 2) cells were transferred to an immobilon filter. The filter was then cut into strips containing β-secretase from each source, and the strips were probed with anti-V5 (Ab) (A), *Ricinus communis* agglutinin (RCA) (B), or *Sambucus nigra* agglutinin (SNA) (C). Bound lectins and antibodies were detected by secondary reactions with alkaline phosphatase-conjugated goat anti-mouse IgG antibody and alkaline phosphatase-conjugated avidin.

ognizes terminal sialic acid, bound only to β-secretases produced by S2βSEC/GalT-ST cells (lane 2C, Fig. 4). However, the recombinant β-secretase produced by S2βSEC cells did not acquire any detectable galactose or sialic acid, indicating that S2βSEC/GalT-ST cells produce recombinant glycoproteins containing both β1,4-linked galactose and α2,6-linked sialic acid. Although further studies will be required to identify the precise structure of the glycosylated β-secretases, stable transformation can be used to add mammalian glycosyltransferases to dipteran insect cells and to directly extend the N-glycosylation pathway of these cells.

3.4. Two-dimensional electrophoresis of recombinant β-secretase from S2βSEC and S2βSEC/GalT-ST cells

The heterogeneity of purified recombinant β-secretases was analyzed by 2D electrophoresis in combination with Western blotting (Fig. 5). Recombinant β-secretases obtained from S2βSEC and S2βSEC/GalT-ST cells were subjected to 2D gel electrophoresis. Six spots, observed from the recombinant β-secretases expressed in S2βSEC cells, were detected in two groups of three spots each in a p*I* range of 4.8–5.7 (Fig. 5A). Purified β-secretases from S2βSEC/GalT-ST cells appeared as 15 spots in a p*I* range of 4.5–5.3 (Fig. 5B). The sialylated form of recombinant β-secretases from S2βSEC/GalT-ST cells had a lower isoelectric point (Fig. 5), similar to the previous finding that the sialic acid residue of the glycoprotein exhibits a p*I* shift to the acidic region, compared to non-sialoglycoprotein (Shimomura and Bremel, 1988; Pirruccello and Lebien, 1985). Recombinant β-secretases from transformed S2 cells also exhibited a high complexity, due to many isoforms and glycosylations.

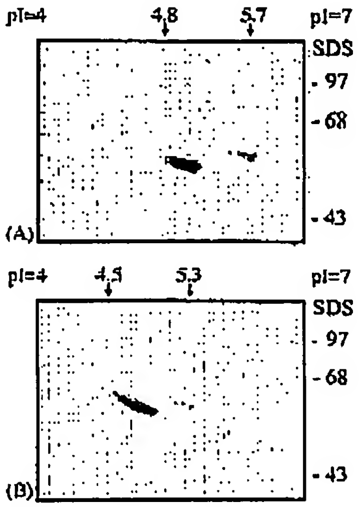

Fig. 5. Two-dimensional gel analysis of recombinant β-secretases from S2βSEC (A) and S2βSEC/GalT-ST (B) cells. Purified β-secretases were separated by p*I* (using Immobiline Drystrip gel, pH 4–7) in the first dimension, followed by SDS-PAGE in the second dimension, then analyzed by Western blot analysis. The p*I* range and molecular weights are indicated.

3.5. Determination of the K_m value, the activity, and IC_{50} value of recombinant β-secretase from S2βSEC and S2βSEC/GalT-ST cells

Extended glycosylation was investigated to determine any affect on the K_m value and the activity of recombinant β-secretases expressed by transformed S2 cells. Recombinant β-secretases were purified from cultures of S2βSEC and S2βSEC/GalT-ST cells by Ni^{2+}-affinity purification. The K_m values of purified β-secretase from S2βSEC and S2βSEC/GalT-ST cells were 4.95 ± 0.03 and 15.84 ± 1.10 μM, respectively.

Recombinant β-secretase from S2βSEC/GalT/ST cells exhibited a lower K_m value compared to control S2βSEC cells, indicating that there is a tighter binding of recombinant β-secretase from S2βSEC/GalT/ST cells to the substrate. Evidently, extended glycosylation modifies the conformation of β-secretase, which results in a different binding affinity for the substrate. The enzyme activities of recombinant β-secretases expressed in S2βSEC and S2βSEC/GalT-ST cells were also estimated (Fig. 6A). Purified β-secretases from S2βSEC cells exhibited relative fluorescence units of 16, 42, 81, 126, and 157 at protein concentrations of 1, 2.5, 5, 7.5, and 10 μg ml^{-1}, respectively, whereas purified β-secretases from S2βSEC/GalT-ST cells exhibited relative fluorescence units of 41, 151, 264, 376, and 464 at the same protein concentrations. The activity of the recombinant β-secretase from S2βSEC/GalT-ST cells was apparently increased up to 260% at a protein concentration of 2.5 μg ml^{-1}. The concentration at half-maximum inhibition values estimated from inhibition analyses using purified β-secretases from S2βSEC and S2βSEC/GalT-ST cells were 11 and 139 nM, respectively (Fig. 6B). The IC_{50} value of recombinant β-secretase from S2βSEC/GalT-ST cells was higher than the IC_{50} value for recombinant β-secretase from control S2βSEC cells, indicating that extended glycosylation might protect recombinant β-secretase from the action of inhibitors. We assumed, based on our lectin blot data and a previous report regarding formation of sialylglycans by baculoviral co-expression of GalT and ST in insect cells (Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001), that the recombinant β-secretase expressed in S2βSEC/GalT-ST cells was sialylated. The activity of the recombinant β-secretase from S2βSEC/GalT-ST cells was probably increased because extended glycosylation apparently leads to the proper conformation of the recombinant protein.

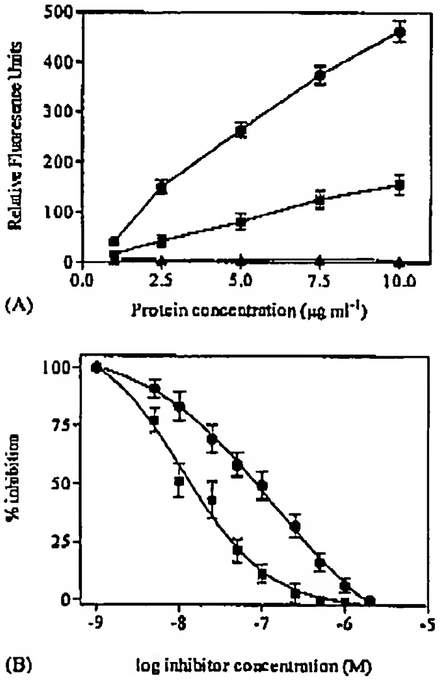

Fig. 6. Activities and inhibition of recombinant β-secretases from S2βSEC and S2βSEC/GalT-ST cells. (A) The enzyme activity in purified preparations of β-secretase from S2βSEC cells (■) and S2βSEC/GalT-ST cells (●), and was measured by a fluorescence-resonance energy-transfer assay. The enzyme activity of unpurified conditioned medium (▲) was also measured as a control. The activity is expressed in relative fluorescence units. (B) The enzyme activity of β-secretase from S2βSEC cells (■) and S2βSEC/GalT-ST cells (●) was measured in the presence and absence of different concentrations of the inhibitor, (Asn^{670}, Sta^{671}, Val^{672})-amyloid β/A4 protein precursor$_{770}$ (662–675).

4. Conclusions

We extended the N-glycan processing capabilities of S2 cells to improve the activity of the glycoprotein β-secretase by transformation of human cDNAs encoding β-secretases and glycosyltransferases (GalT and ST) in the S2 cell line. Western blot analysis indicated that the molecular weights of recombinant β-secretase were increased in transformed S2βSEC/GalT-ST cells. Based on a lectin blot analysis, recombinant β-secretase from S2βSEC/GalT-ST cells contained the galactose and terminal sialic acid in the oligosaccharide. The enzyme activity of β-secretase from S2βSEC/GalT-ST cells was enhanced 2.6 times. To the best of our knowledge, we report for the first time an enhanced activity of recombinant β-secretase expressed in S2 cells co-transformed with human cDNAs coding glycosyltransferases. This method can produce more authentic recombinant glycoproteins with complex N-linked glycans containing a penultimate galactose and a terminal sialic acid.

Acknowledgements

The authors thank Dr. Colin Dingwall for providing the human β-secretase cDNA construct. This work was supported by a grant (R05200300011149702003) from the Korea Science and Engineering Foundation and also by grants from Dong-Young ENG, and from the Korea Science and Engineering Foundation through the Plant Metabolism Research Center, Kyung Hee University.

References

Ailor, E., Takahashi, N., Tsukamoto, Y., Masuda, K., Rahman, A.A., Jarvis, D.L., Lee, Y.C., Betenbaugh, M.J., 2000. N-glycan patterns of human transferrin produced in *Trichoplusia ni* insect cells: effects of mammalian galactosyltransferase. Glycobiology 10, 837–847.

Breitbach, K., Jarvis, D.L., 2001. Improved glycosylation of a foreign protein by Tn-5B1-4 cells engineered to express mammalian glycosyltransferases. Biotechnol. Bioeng. 74, 230–239.

Bruinzeel, W., Yon, J., Giovannolli, S., Masure, S., 2002. Recombinant insect cell expression and purification of human β-secretase (BACE-1) for X-ray crystallography. Protein Express. Purif. 26, 139–148.

Chang, G.D., Chen, C.J., Lin, C.Y., Chen, H.C., Chen, H., 2003. Improvement of glycosylation in insect cells with mammalian glycosyltransferase. J. Biotechnol. 102, 61–71.

Charlwood, J., Dingwall, C., Matico, R., Hussain, I., Johanson, K., Moore, S., Powell, D.J., Skehel, J.M., Ratcliffe, S., Clarke, B., Trill, J., Sweitzer, S., Camilleri, P., 2001. Characterization of the glycosylation profiles of Alzheimer's beta-secretase protein Asp-2 expressed in a variety of cell lines. J. Biol. Chem. 276, 16739–16748.

Glenner, G.G., Wong, C.W., 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. 120, 885–890.

Haass, C., Hung, A.Y., Selkoe, D.J., Teplow, D.B., 1994. Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid beta-protein precursor. J. Biol. Chem. 269, 17741–17748.

Heo, J., Staples, C.R., Ludden, P.W., 2001. Redox-dependent CO_2 reduction activity of CO dehydrogenase from *Rhodospirillum rubrum*. Biochemistry 40, 7604–7611.

Hollister, J., Jarvis, D.L., 2001. Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1,4-galactosyltransferase and alpha 2,6-sialyltransferase genes. Glycobiology 11, 1–9.

Jarvis, D.L., Finn, E.E., 1995. Biochemical analysis of the N-glycosylation pathway in baculovirus-infected lepidopteran insect cells. Virology 212, 500–511.

Jarvis, D.L., Finn, E.E., 1996. Modifying the insect cell N-glycosylation pathway with immediate early baculovirus expression vectors. Nat. Biotechnol. 14, 1288–1292.

Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., Muller-hill, B., 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325, 733–736.

Kim, H.G., Yang, S.M., Lee, Y.C., Do, S.I., Chung, I.S., Yang, J.M., 2003. High-level expression of human glycosyltransferases in insect cells as biochemically active form. Biochem. Biophys. Res. Commun. 305, 488–493.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage. Nature 227, 680–685.

Mallender, W.D., Yager, D., Onstead, L., Nichol, M.R., Eckman, C., Sambamurti, K., Kopcho, L.M., Marcinkeviciene, J., Copeland, R.A., Rosenberry, T.L., 2001. Characterization of recombinant, soluble beta-secretase from an insect cell expression system. Mol. Pharmacol. 59, 619–626.

Marz, L., Altmann, F., Staudacher, E., Kubelka, V., 1995. Protein glycosylation in insects. In: Montreuil, J., Schachter, H., Vliegenthart, J.F.G. (Eds.), Comprehensive Biochemistry: Glycoproteins. Elsevier Science, Amsterdam, pp. 543–563.

Park, J.H., Chang, K.H., Lee, J.M., Lee, Y.H., Chung, I.S., 2001. Optimal production and in vitro activity of recombinant endostatin from stably transformed *Drosophila melanagaster* S2 cells. In Vitro Cell. Dev. Biol. 37, 5–9.

Pasternak, J.J., 1993. DNA sequence organization and gene copy number determination. In: Glick, B.R., Thompson, J.E. (Eds.), Methods in Plant Molecular Biology and Biotechnology. CRC Press, Inc., Ann Arbor, pp. 29–36.

Pirruccello, S.J., Lebien, T.W., 1985. Monoclonal antibody BA-1 recognizes a novel human leukocyte cell surface sialoglycoprotein complex. J. Immunol. 134, 3962–3968.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, New York.

Selkoe, D.J., 1991. The molecular pathology of Alzhimer's disease. Neuron 6, 487–498.

Shimomura, K., Bremel, R.D., 1988. Characterization of bovine placental lactogen as a glycoprotein with N-linked and O-linked carbohydrate side chain. Mol. Endocrinol. 2, 845–853.

Tomiya, N., Betenbaugh, M.J., Lee, Y.C., 2003. Humanization of lepidopteran insect-cell-produced glycoproteins. Acc. Chem. Res. 36, 613–620.

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EXHIBIT B

Boog *et al.*, entitled "Specific immune responses restored by alteration in carbohydrate chains of surface molecules on antigen presenting cells," *Eur. J. Immunol.* **19**: 537-542, 1989

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Specific immune responses restored by alteration in carbohydrate chains of surface molecules on antigen-presenting cells*

Two class I major histocompatibility (MHC) mutant mouse strains, $H\text{-}2^{bm14}$ and $H\text{-}2^{bm6}$, differ from the strain of origin C57BL/6 (B6, $H\text{-}2^{b}$) in one and two amino acids of the $H\text{-}2D^{b}$ and $H\text{-}2K^{b}$ molecule, respectively. The bm14 D^{b} mutation results in specific failure of female bm14 mice to generate a cytotoxic T lymphocyte (T_c) response to the male-specific antigen H-Y. The allospecific T_c response of $CD8^{+}$ B6 T cells against bm6 K^{b} mutant spleen cells, in contrast to that against other K^{b} mutants, is absolutely $CD4^{+}$ T helper cell dependent. Purified $CD8^{+}$ T cells completely fail to respond. We now report that the inability to mount these specific immune responses is restored by the use of dendritic cells (DC) as antigen-presenting cells (APC). Comparison of MHC expression on various types of APC by cytofluorimetry and quantitative immunoprecipitation showed very high expression of class I and class II MHC molecules on DC. Strikingly, examination of class I and class II molecules by isoelectric focusing revealed qualitative differences as well. We show that the surface MHC class I molecules of DC are present in greater quantity and carry on average fewer sialic acids than the same molecules isolated from other APC types such as spleen cells, lipopolysaccharide blasts or concanavalin A blasts. That sialic acids on cell surface molecules, including MHC, may play a role in antigen presentation is suggested by our finding that removal of sialic acids, by neuraminidase, can restore specific responses to nonresponder APC as well.

1 Introduction

T lymphocyte responses against specific antigens are regulated by class I and class II molecules encoded by the major histocompatibility complex (MHC) [1-4]. Failure of T cells to respond to a given antigen has been explained along two main lines, lack of appropriate antigen presentation or, alternatively, deletions in the T cell repertoire [5-7]. It has been proposed recently that the interaction between MHC molecules and antigens is a major constraint [8-10]. Among many accessory cell types carrying both class I and class II antigen, dendritic cells (DC) possess an exceptionally strong stimulating capacity to induce allospecific proliferative T helper (T_h) cell responses to class II MHC antigen [11, 12] and allospecific cytotoxic T cell (T_c) responses to class I MHC antigens [12]. We have previously demonstrated that DC can be used to overcome several specific immune response defects [13-16]. However, the unique characteristics of DC as antigen-presenting cells (APC) have thus far defied a molecular explanation. Evidence from different groups has suggested that T cell recognition of stimulator cells may be influenced by the state of glycosylation of the stimulator cells [17-21]. The class I $H\text{-}2D^{b}$ mutant mouse strain bm14 differs in one amino acid ($Gln^{70} \rightarrow His$) from the parental strain (C57 BL/6; B6; $H\text{-}2^{b}$) [22]. As a consequence bm14 female mice are unable to mount a T_c response against the male specific antigen HY. The class I $H\text{-}2K^{b}$ mutant bm6 differs in two amino acids ($Tyr^{116} \rightarrow Phe$; $Cys^{121} \rightarrow Arg$) from the parental strain B6 [23]. With bm6 spleen cells (SC) as stimulator cells the $CD8^{+}$ subset of B6-responding cells do not produce interleukin 2 (IL 2). As a result the B6 anti-bm6 T_c response is strictly $CD4^{+}$ T_h cell dependent, in contrast to the response of B6 T cells against other K^{b} mutants [24]. In the present study we first examined whether the inability to mount these specific immune responses could be restored by the use of DC as APC. Whereas SC were indeed unable to present antigen, DC stimulated the specific responses efficiently. Our results from comparison of different types of APC by cytofluorimetry and biochemical analysis show that DC not only carry a larger amount of class I and class II MHC, but also that the MHC molecules on DC on average carry fewer sialic acids.

[I 7059]

2 Materials and methods

2.1 Animals and immunization

All mice used in this study were bred at the Netherlands Cancer Institute. The C57BL/6 Kh (B6, $H\text{-}2^{b}$), B6 $C\text{-}H\text{-}2^{bm14}$ (bm14) and B6 $H\text{-}2^{bm6}$ (bm6) mice have been described before [14-16]. For priming against the male antigen H-Y, female mice were primed by one i.p. injection of 10^{5} male DC [13].

2.2 Isolation of DC

DC were isolated by the Steinman protocol [25] with minor modifications as described [13]. Briefly, SC were spun on a

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Abbreviations: APC: Antigen-presenting cell(s) Con A: Concanavalin A DC: Dendritic cell(s) IEF: Isoelectric focusing Ir: Immune response LPS: Lipopolysaccharide mAb: Monoclonal antibody(ies) MHC: Major histocompatibility complex NANAse: Neuraminidase rIL 2: Recombinant interleukin 2 SC: Spleen cells T_c: Cytotoxic T lymphocyte T_h: T helper lymphocyte

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discontinuous bovine serum albumin gradient of 10%, 28% and 35% BSA (ρ = 1.099, 1.080 and 1.031 kg/l) for 30 min at 10000 × g. The interphase (alpha band) 10%–28% was removed and cultured for 90 min in glass petri dishes. Nonadherent cells were discarded and the medium was replaced. After a further 18 h of culture, nonadherent cells were separated by Fc receptor (FcR) rosetting. FcR^- cells (≈ DC) were then separated from FcR^+ cells over a Lympho-cyte-M. cushion (Cedarlane Labs., Hornby, Ontario, Canada).

2.3 Depletion of T cell subpopulations

Fractionated $Lyt2^+$ ($CD8^+$) or $L3T4^+$ ($CD4^+$) T cell subpopulations were obtained from nylon wool-passed SC of primed (bm14 H-Y specific) or unprimed (anti-class I H-2 K^{bm6} specific) mice by double treatment with either anti-Ly-2.2 (1:1000 dilution of ascites fluids, NEN, Boston, MA) or anti-L3T4 (1:40 dilution of hybridoma culture supernatant, SN 172-4, a gift from Dr. H. R. MacDonald) monoclonal antibodies (mAb) and complement (1:10 Low Tox-M rabbit complement C, Cedarlane) as described previously [13, 14]. Efficacy of treatment (> 99%) was confirmed by flow microfluorimetry.

2.4 Induction and measurement of T_c responses

H-Y specific and K^{bm6} specific T_c cells were generated, using different responder T cell subpopulations as described previously [13]. Briefly, untreated or fractionated responder bm14 H-Y-primed or unprimed B6 cells (10^6) were cultured with either irradiated (2500 rad) (male) SC 10^6, (male) SC (10^6) treated with neuraminidase (NANAse) or (male) DC (5 × 10^4) stimulator cells in 200 µl of culture medium in 96-well roundbottom microtiter plates (Flow Laboratories, Rickmansworth, GB). Stimulator cells were treated with NANAse (*Clostridium perfringens* type VIII, Sigma Biochemical Co., St. Louis, MO) according to the method of Cowing and Chapdelaine [19]. In brief, cells were incubated at 5 × 10^7/ml in Iscove's medium containing 2U NANAse for 30 min at 37 °C, washed and used for culture. After five days of culture at 37 °C in humidified air with 5% CO_2, various dilutions of effector cells were tested on ^{51}Cr-labeled (male) target cells [5 × 10^3, lipopolysaccharide (LPS) blasts]. The recombinant interleukin (IL) 2 (rIL 2) for these studies was kindly supplied by Biogen S.A. (Geneva, Switzerland) and used at a final concentration of 50 U/ml.

2.5 Flow cytofluorimetric analysis

The mAb used and their known specificities have been described previously [26–28]. Aliquots of 5 × 10^5 untreated or treated cells in 100 µl were incubated at 4 °C with biotinylated mAb [28]: anti-class I (anti-H-2 D^b B22-249R1 [29] or anti-H-2 K^b K7-65 [27] and anti-class II (anti I-$A^{k,b,d,r}$, 17/227 [28]). After 30 min the cells were washed and 25 µg/ml avidin-fluorescein conjugate (Becton Dickinson, Sunnyvale, CA) was added for an additional 30 min at 4 °C. Control samples were stained with second-step reagent only. Cells were washed three times and all samples were passed through a flow cytometer (FACS IV, Becton Dickinson).

2.6 Biochemical analysis of MHC class I and class II molecules at the surface of APC

APC were radio-iodinated by the lactoperoxidase method [30]. Nonidet-P40 (NP40) lysates were prepared and subjected to either two-dimensional (2D) gel electrophoresis or one-dimensional (1D) isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For 2D-gel electrophoresis [31] NP40 lysates of surface-iodinated cells were split and one half was subjected to NANAse treatment as described [32]. Lysates were analyzed by 2D-gel electrophoresis as described [32]. For 1D IEF and SDS-PAGE, class I antigens were immunoprecipitated out of NP-40 lysates as described [32–34], with a rabbit anti-H-$2K^b$ serum, broadly reactive with murine class I MHC molecules, kindly provided by Dr. S. G. Nathenson, Albert Einstein College of Medicine, Bronx, N.Y. For quantitative gel electrophoresis class I antigens were immunoprecipitated out of a constant amount of trichloroacetic acid (TCA)-precipitable counts and the immunoprecipitates analyzed by 12% SDS-PAGE [35]. Quantitation of the radioactive bands as excised from the gel was performed as described [35]. For 1D-IEF analysis constant amounts of immunoprecipitated material were loaded on 1D-IEF with or without previous NANAse digestion as described [32].

3 Results

3.1 Restoration of antigen-specific T_c responses by DC or SC treated with NANAse as APC

Responder spleen cells (unfractionated or depleted of $CD4^+$ cells) from either H-Y-primed bm14 female mice or unprimed normal B6 mice were restimulated *in vitro* with different types of APC. The resulting cytolytic activities against (male) target cells are shown in Fig. 1. The left panel shows that T cells from H-Y-primed bm14 female mice failed to kill male target cells after restimulation with normal male SC (as expected [36]), yet responded when restimulated with bm14 DC, in line with our previous results [14, 15]. The right panel of Fig. 1 shows that the failure of $CD8^+$ B6 cells to respond (as expected [24]) could be overcome by stimulation with DC of bm6 mice. After treatment of spleen cells with NANAse, both the bm14 anti-H-Y response and the B6 ($CD8^+$) anti-bm6 response were restored to levels observed by responder cells stimulated with (untreated) DC, or SC supplemented with rIL 2. This level of response is achieved with 10^6 NANAse-treated spleen cells and as few as 5 × 10^4 DC, whereas 5 × 10^4 NANAse-treated spleen cells do not stimulate a response (data not shown).

3.2 Flow microfluorimetric analysis of untreated or NANAse-treated stimulator cells

Upon comparison of different types of APC from B6 mice for MHC expression by cytofluorimetry, DC showed very high expression of both class I and class II MHC antigens (Fig. 2). Different cell suspensions were analyzed with and without prior treatment with NANAse. Whereas no shift in mean fluorescence index was observed for DC as a consequence of this treatment, SC, LPS blasts and Con A blasts showed significantly more intense staining after removal of sialic acids. These findings indicate that the epitopes on the latter cell types seen by the B22-249R1 (anti-H-2 D^b) and 17/227 (anti-I-

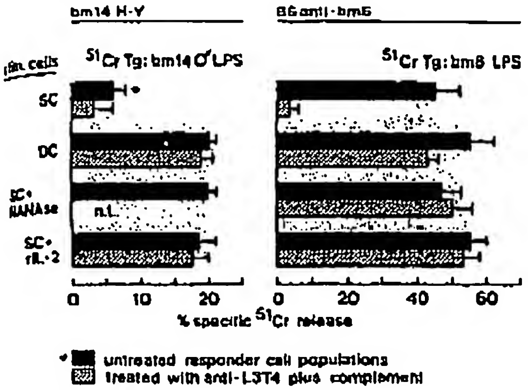

Figure 1. Restoration of antigen-specific T_c responses by antigen presentation with DC or SC treated with NANAse. Unfractionated or fractionated CD8$^+$ responder T cell subpopulations (10^6) were cultured with either irradiated (2500 rad) (male) SC (10^6), (male) SC (10^6) treated with NANAse or (male) DC (5×10^4) stimulator cells. After 5 days various dilutions of effector cells were tested on ^{51}Cr-labeled (male) target cells (5×10^3 LPS blasts). The results shown are those obtained at a responder cell concentration of 10^6 (mean of five experiments and SEM indicated by bars). These responses are specific because lysis of bm14 female (bm14 anti-H-Y response) or B6 (B6 anti-bm6 response) target cells was always less than 6%, and restimulation of unfractionated B6 responder cell populations with irradiated syngeneic B6 stimulator cells (SC, LPS and Con A blasts) treated with NANAse did not result in lysis of bm6 target cells (data not shown). The rIL 2 for these studies was used at a final concentration of 50 U/ml.

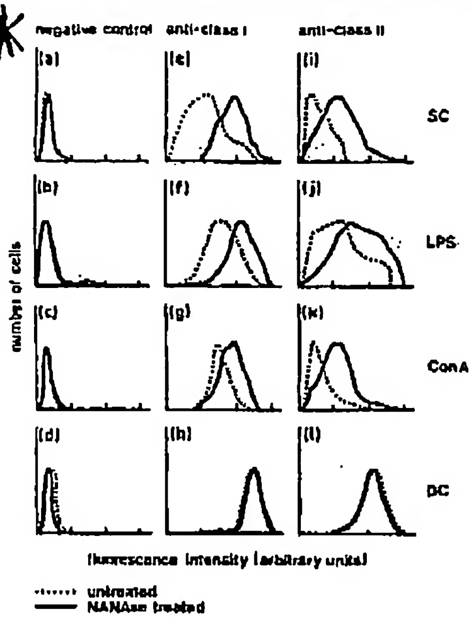

Figure 2. Flow microfluorimetric analysis of untreated (dotted lines) or NANAse-treated (solid lines) stimulator cells: SC, LPS blasts, Con A blasts and DC. Linear fluorescence histograms are expressed in arbitrary units. NANAse treatment was carried out as described in Sect. 2.4. Aliquots of 5×10^5 untreated or treated cells in 100 µl were incubated at 4 °C with the following biotinylated mAb: (a–d) no antibody; (e–h) anti-class I (anti-D^b B22-249R1 [26]), similar results were obtained with anti-K^b K7-65 [27] (data not shown), (i-l) anti-class II (anti-I-A^{b,k,d,r}, 17/227 [28]). After 30 min the cells were washed and 25 µg/ml avidin-fluorescein conjugate (Becton Dickinson) was added for an additional 30 min at 4 °C. Control samples were stained with second-step reagent only. Cells were washed three times and all samples were passed through the flow cytometer FACS IV.

A^b) antibodies are rendered more accessible by NANAse treatment. Similar results were obtained with anti-K7-65 (anti-H-2 K^b) mAb (data not shown). A logical assumption from these studies is that MHC molecules on DC carry less sialic acids than the other APC types as indeed proved to be the case upon biochemical analysis.

3.3 Biochemical analysis of class I and II MHC molecules on different types of B6 APC

To further explore quantitative and qualitative aspects of MHC expression in B6 APC, including sialic acid content, these cells were subjected to biochemical analysis. First the total amount of cell surface MHC class I molecules was determined by quantitative immunoprecipitation with a broadly reactive polyclonal rabbit anti-mouse class I serum. This method of quantitation is independent of sialic acid content of MHC molecules as proven by the fact that treatment of radiolabeled lysates with NANAse prior to immunoprecipitation did not increase the yield of precipitated class I antigens (data not shown). In contrast to the results obtained with all APC types except DC in cytofluorimetry (see Sect. 3.2), DC expressed by far the largest surface amounts of class I antigens in comparison with the other B6 APC types (Fig. 3C). Per constant amount of precipitable counts and setting the amount of class I heavy chains in SC at the arbitrary value of 1.0, Con A blasts contained 4.8, LPS blasts 2.7 and DC 12.5 times as much class I antigens.

Strikingly, examination of class I antigens by IEF revealed qualitative differences as well. Class I molecules isolated from lysates of radio-iodinated DC from B6 mice contained on average 2–3 molecules of sialic acid per molecule of heavy chain, whereas on B6 LPS blasts, Con A blasts, or unstimulated SC an average of 4 sialic acids per molecule of heavy chain were present (Fig. 3A, B). Similar results were obtained for each subunit of I-A^b class II MHC antigen of B6 cells and for class I MHC antigens of bm6 cell populations (data not shown). Therefore, the class I and class II molecules on DC isolated from mouse spleen carry less sialic acid than those on other types of APC.

A pulse-chase experiment performed on class I antigens from DC revealed that this lower extent of sialylation is acquired biosynthetically as a consequence of reduced sialylation and is not a consequence of the action of neuraminidases at the cell surface (data not shown).

4 Discussion

The salient findings of this study are (a) MHC expression on DC is both quantitatively superior and qualitatively distinct from that on other APC types. The qualitative difference lies in the presence of fewer sialic acids on MHC molecules on DC than on the other tested APC. (b) This lower degree of sialyla-

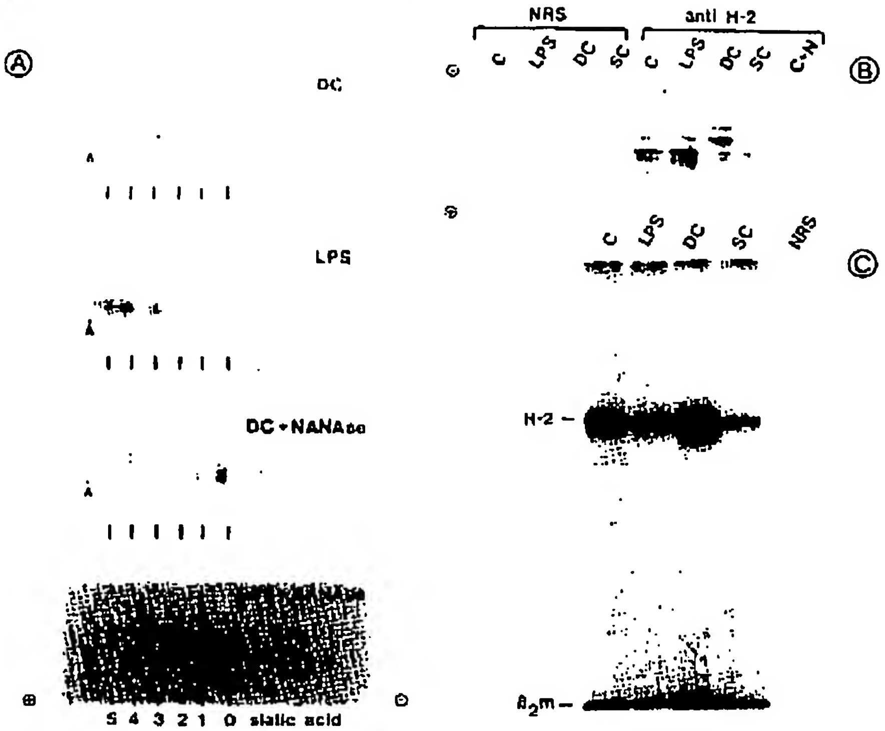

Figure 3. Biochemical analysis of class I molecules from different types of APC from B6 mice. (A) Differences in sialylation are visible in total cell lysates of APC. Total lysates of DC or LPS blasts were subjected to 2D gel electrophoresis prior to and after digestion of the lysate with NANAse. Only the region of the gel around 45 kDa is shown. The arrowhead indicates the position of migration of actin. The major radiolabeled species in all panels are the class I heavy chains, as verified by immunoprecipitation (not shown). After digestion with NANAse, class I heavy chains from DC and LPS blasts occupy the same (more basic) position on the gel. Anode (+) is to the left. (B) Class I heavy chains on DC carry less sialic acids than those on other APC. Lysates prepared from radio-iodinated cells were subjected to immunoprecipitation with a rabbit anti-H-2K^b serum (anti-H-2), provided by Dr. S. Nathenson, or normal rabbit serum (NRS). Lysates from Con A blast (C), LPS blasts (LPS), DC or SC were used as starting material. The resulting immunoprecipitates were analyzed by 1D-IEF [32]. In addition, an aliquot of class I antigens precipitated from Con A blasts was analyzed after digestion with NANAse (C + N). Note the different distribution of sialylated class I heavy chains for DC, as compared to the other cell types. The amounts of immunoprecipitate loaded for DC were matched for radioactivity with those for C and LPS. Only the region of the class I heavy chain is shown. Mouse β_2-microglobulin (β_2m) migrates near the very top (basic end) of this type of IEF gel [33, 34]. (C) Determination of the quantity of class I antigens in different types of APC. Lysates were prepared from iodinated APC. Amounts of radioactivity present in the lysates were determined by TCA precipitation. Equal amounts of radioactivity were then subjected to immunoprecipitation, using the rabbit anti-H-2 serum, or normal rabbit serum (NRS: shown for Con A blasts). The resulting immunoprecipitates were analyzed by SDS-PAGE on a 12% gel followed by autoradiography. The class I H-chains, localized after autoradiography, were excised and their radioactivity determined. Setting the amount of heavy chains in SC at the arbitrary value of 1.0, the following relative amounts were calculated. Con A blasts: 4.8; LPS blasts: 2.7; DC: 12.5. It is assumed that the cell surfaces of these different types of cells do not differ significantly in their ability to be radio-iodinated.

tion may contribute to the superior antigen-presenting function of DC, since NANAse treatment of nonresponder types of APC restores specific failure of T cells to respond to nominal antigen or alloantigen. These results do not prove, however, that only sialic acid attachment to MHC molecules is relevant for APC function. Below we shall discuss each of these points in more detail.

The high density of class I MHC molecules on DC found by cytofluorimetry in our study confirms a previous report based on binding of radiolabeled class I-specific antibody [37]. Our quantitative immunoprecipitation results provide unequivocal biochemical evidence independent of the extent of glycosylation of MHC molecules, that class I expression on DC is indeed quantitatively superior in comparison with the other APC types tested. This biochemical confirmation of the results obtained in binding studies is important because our results clearly show that extent of sialylation strongly affects binding of anti-class I and II mAb (FACS analysis, Fig. 2) as found previously by others [38]. Similar findings pertaining to the binding of anti-human MHC antibodies on control and NANAse-treated cells have been obtained in our laboratory*.

It was shown earlier that antigen presentation (by B cells) is improved by NANAse treatment as measured by proliferation in mixed lymphocyte culture [19]. It was suggested that differential glycosylation of class II MHC molecules could explain the difference in MHC stimulatory potential between resting B cells and B cell blasts [19]. Several additional reports also stress the influence of glycosylation on APC function [17, 18,

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* Rotteveel, Neefjes, Ploegh and Lucas, submitted.

21]. One can therefore pose the question to which extent the sparsely sialylated state of MHC molecules, occurring naturally in DC, contributes to the superior APC function of these cells. At present there is no simple answer to this question. First of all it seems likely that the low degree of sialylation is not confined to MHC molecules, there being no reason for selectively low sialylation of MHC only. Rather we hypothesize that DC have a generally low sialylation state of their cell surface molecules including T200, that is abundantly expressed on DC [37] and hitherto poorly studied structures on DC, such as counterstructures of adhesion molecules like LFA-1. This would create a low net negative charge at the surface of DC, which, together with the long dendritic projections of these cells, might explain their pronounced tendency to cluster formation with T cells, that is considered so important for APC function [39]. Indeed we observed that the NANAse-treated SC APC capable of restoring the response of bm14 T cells to H-Y and of CD8$^+$ B6 T cells to the bm6 mutant, also show markedly enhanced cluster formation with the responding T cells (data not shown).

SC spleen cells

In the restoration of the bm14 T_c response to H-Y presented on DC, the level of D^{bm14} glycosylation may be directly relevant for APC function. "Native D^b-like" epitopes are serologically demonstrable on bm14 DC by mAb binding at the same very high binding level as on B6 DC. On bm14 SC, in contrast to B6 SC, these epitopes are hardly detectable [15]. Likewise, priming of bm14 female mice with bm14 male DC allows the expansion of a "native D^b-like" T_c repertoire not seen upon priming with male SC [15]. Thus on the D^{bm14} molecule of DC, D^b-like determinants are accessible to D^b-specific mAb and D^b-restricted T_c cells, that are not accessible on bm14 SC. This may be the result of the qualitative difference between the D^{bm14} molecules in these cell types [15].

If class I glycosylation is considered to be involved in antigen presentation, the three-dimensional structure of class I MHC [40, 41] must be contemplated. The N-linked glycosylation sites present in K^b and D^b heavy chains include attachment sites at positions 86 and 176. These residues occur at the extremities of the two α helices that make an essential contribution to the formation of the putative binding site for (processed) antigen. The close proximity of these two glycans to the site which binds antigen suggests the possible interference of sialic acids with binding of processed antigen to MHC molecules, and/or with the recognition by the T cell receptor of the complex between MHC molecule and processed antigen. Our observations indicating increased binding of class I α1 domain specific mAb, as shown by FACS (Fig. 2) in the face of constant amounts of total class I antigen, as determined biochemically, show that removal of sialic acid can indeed improve accessibility of the class I molecule to "receptors" (immunoglobulin or T cell receptor) that recognize them [42]. Increased binding of MHC class I-specific mAb to mouse lymphoblasts following influenza virus infection was attributed to viral neuraminidase and likewise proven to be due to an increase in affinity rather than an increase in the number of binding sites [38]*. Alternatively, differential glycosylation of MHC class I may affect the interaction between the CD8 molecule on T_c cells and class I MHC. Obviously, at this stage, involvement of MHC glycosylation in APC function does not rule out an additional or even predominant effect of glycosylation of other surface molecules on APC function.

After NANAse treatment the response is no longer dependent on exogenous rIL 2. This suggests that the response restoration by DC or by NANAse-treated SC operates by lowering the threshold for IL 2 production by T cells, CD8$^+$ T cells in particular, thereby decreasing or obviating the need for CD4$^+$ T_h cells in T_c responses. Direct activation of CD8$^+$ T_c responses by DC has indeed been documented in a variety of class I MHC allospecific and class I-restricted T_c responses [14, 43]. Moreover, this concept implies that defective IL 2 production by CD8$^+$ cells in the B6 anti-bm6 T_c response [24] is restored by stimulation with DC or NANAse-treated SC. Preliminary data indicate that this is indeed the case (unpublished observations). Presumably, the more powerful stimulus provided by these APC is responsible for this restoration. A recent study indicates that class I MHC-reactive T cells clonally segregate into two functionally distinct subsets: IL 2-producing and cytolytic cells with minor (< 10%) overlap [44]. We postulate that DC and NANAse-treated SC either more efficiently activate the IL 2-secreting noncytolytic subset of CD8$^+$ cells, or induce IL 2 secretion in CD8$^+$ T cells with actual lytic ability.

Because both DC and NANAse-treated SC as APC are capable of restoring the response defects studied, one might ask what the functional significance is of the greater quantity of class I MHC on DC in comparison with SC. In this regard it is important to stress that stimulation with 10^6 NANAse-treated SC stimulates a T_c response comparable to that observed with 5×10^4 DC, confirming our recent study on the presentation of viral antigens in which, on a per cell basis, DC are at least 10-fold more efficient in APC function than other APC types [16]. A recent study with cell-sized artificial membranes also indicates the importance of class I MHC density [45].

Restoration of specific T cell response defects by DC or NANAse-treated SC is not always achieved. For example the failure of the bm1 K^b mutant to generate a T_c response against Sendai virus [46] is not restored by these procedures [16]. This defect could therefore be based on complete failure of the K^{bm1} molecule to associate with a relevant Sendai virus peptide or on a true T_c repertoire defect.

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5 References

1 Paul, W. E. in Paul, W. E. (Ed.), *Fundamental Immunology*, Raven Press, New York 1984, p. 439.
2 Simpson, E. and Gordon, R. D., *Immunol. Rev.* 1977. *35:* 59.
3 Von Boehmer, H., Fathman, C. G. and Haas, W., *Eur. J. Immunol.* 1977. *7:* 443.
4 Melief, C. J. M., Stukart, M. J., De Waal, L. P., Kast, W. M. and Melvold, R. W., *Transplant. Proc.* 1983. *15:* 2086.
5 Nagy, Z. A., Baxevanis, C. N., Ishii, N. and Klein, J., *Immunol. Rev.* 1981. *60:* 59.
6 Rosenthal, A. S., Barcinsky, M. A. and Blake, J. T., *Nature* 1977. *267:* 156.
7 Schwartz, R. H., *Scand. J. Immunol.* 1978. *7:* 3.
8 Ogasawara, K., Maloy, W. M. and Schwartz, R. H., *Nature* 1987. *325:* 450.
9 Guillet, J. G., Lai, M.-Z., Briner, T. J., Buus, S., Sette, A., Grey, H. M., Smith, J. A. and Gefter, M. L., *Science* 1987. *235:* 865.
10 Allen, P. M., Matsueda, G. R., Evans, R. J., Dunbar, J. B., Marschall, G. R. and Unanue, E. R., *Nature* 1987. *327:* 713.
11 Steinman, R. M. and Witmer, M. D., *Proc. Natl. Acad. Sci. USA* 1978. *75:* 5132.

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* Rotteveel, Neefjes, Ploegh and Lucas, submitted.

12 Steinman, R. M. and Nussenzweig, V., *Immunol. Rev.* 1980. *53:* 127.
13 Boog, C. J. P., Kast, W. M., Timmers, H. Th. M., Boes, J., De Waal, L. P. and Melief, C. J. M., *Nature* 1985. *318:* 59.
14 Boog, C. J. P., Boes, J., Melief, C. J. M., *Eur. J. Immunol.* 1988. *18:* 219.
15 Boog, C. J. P., Boes, J., Melief, C. J. M., *J. Immunol.* 1988. *140:* 3331.
16 Kast, W. M., Boog, C. J. P., Roep, B. O., Voordouw, A. C. and Melief, C. J. M., *J. Immunol.* 1988. *140:* 3186.
17 Hart, G. W., *J. Biol. Chem.* 1982. *257:* 151.
18 Cullen, S. E., Kindle, C. S., Shreffler, D. C. and Cowing, C., *J. Immunol.* 1981. *127:* 1478.
19 Cowing, C. and Chapdelaine, J. M., *Proc. Natl. Acad. Sci. USA* 1983. *80:* 6000.
20 Tabira, S., Kakiuchi, T., Minotti, M. and Nariuchi, H., *J. Immunol.* 1986. *137:* 2448.
21 Powell, L. D., Whiteheart, S. W. and Hart, G. W., *J. Immunol.* 1987. *139:* 262.
22 Hemmi, S., Geliebter, J., Zeff, R. A., Melvold, R. W. and Nathenson, S. G., *J. Exp. Med.* 1988. *168:* 2319.
23 Nathenson, S. G., Geliebter, J., Pfaffenbach, G. M. and Zeff, R. A., *Annu. Rev. Immunol.* 1986. *4:* 471.
24 Rosenberg, A. S., Mizuochi, T. and Singer, A., *Nature* 1986. *322:* 829.
25 Steinman, R. M., Kaplan, G., Witmer, M. D. and Cohn, Z., J. Exp. Med. 1979. *149:* 1.
26 Hämmerling, G. J., Hämmerling, U. and Lemke, H., *Immunogenetics* 1979. *8:* 433.
27 Hämmerling, G. J., Rusch, E., Tada, N., Kimura, S. and Hämmerling, U., *Proc. Natl. Acad. Sci. USA* 1982. *79:* 4737.
28 Lemke, H., Hämmerling, G. F. and Hämmerling, U., *Immunol. Rev.* 1979. *47:* 175.
29 Wormmeester, J. F., Stiekema, F. and De Groot, K., *J. Immunol. Methods* 1984. *67:* 389.
30 Phillips, D. R. and Morrison, M., *Biochem. Biophys.* *mun.* 1970. *40:* 284.
31 O'Farrell, P. H., *J. Biol. Chem.* 1975. *250:* 2007.
32 Neefjes, J. J., Breur-Vriesendorp, B. S., Van Seven
Ivanyi, P. and Ploegh, H. L., *Human Immunol.* 1986.
33 Krimpenfort, P., Rudenko, G., Hochstenbach, F., G
Berns, A. and Ploegh, H., *EMBO J.* 1987. *6:* 1673.
34 Rein, R. S., Seemann, G. H. A., Neefjes, J. J., Hochs
M. H., Stam, N. J. and Ploegh, H. L., *J. Immunol.*
1178.
35 Neefjes, J. J. and Ploegh, H. L., *Eur. J. Immunol.* 19
36 Steinman, R. M. and Nussenzweig, V., *Immunol. Re*
127.
37 Nussenzweig, M. C., Steinman, R. M., Unkeless, J. C
M. D., Gutchinov, B. and Cohn, Z. A., *J. Exp. Med*
168.
38 Liberti, P. A., Hackett, C. J. and Askonas, B. A., *Eu*
nol. 1979. *9:* 751.
39 Steinman, R. M., Inaba, K., Schuler, G., Witmer, M.
S., Flechner, E., Bhardwaj, N. and Young, J. W., in
and Miller R. G., (Eds), *Progr. Immunol.* 1986. *VI:* 1
40 Bjorkman, P. J., Saper, M. A., Samraoui, B., Benr
Strominger, J. L. and Wiley, D. C., *Nature.* 1987. *32*
41 Bjorkman, P. J., Saper, M. A., Samraoui, B., Benr
Strominger, J. L. and Wiley, D. C., *Nature* 1987. *329*
42 Schauer, R., *Trends Biochem. Sci.* 1985. *10:* 357.
43 Inaba, K., Young, J. W. and Steinman, R., *J. Exp.*
166: 182.
44 Heeg, K., Steeg, C., Hardt, C. and Wagner, H., *Eur.*
1987. *17:* 229.
45 Goldstein, S. A. N. and Mescher, M. F., *J. Immunol*
2034.
46 De Waal, L. P., Kast, W. M., Melvold, R. W. and N
M., *J. Immunol.* 1983. *130:* 1090.

EXHIBIT C

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Review

Glycoproteins from Insect Cells: Sialylated or Not?

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Our growing comprehension of the biological roles of glycan moieties has created a clear need for expression systems that can produce mammalian-type glycoproteins. In turn, this has intensified interest in understanding the protein glycosylation pathways of the heterologous hosts that are commonly used for recombinant glycoprotein expression. Among these, insect cells are the most widely used and, particularly in their role as hosts for baculovirus expression vectors, provide a powerful tool for biotechnology. Various studies of the glycosylation patterns of endogenous and recombinant glycoproteins produced by insect cells have revealed a large variety of O- and N-linked glycan structures and have established that the major processed O- and N-glycan species found on these glycoproteins are (Galβ1,3)GalNAc-O-Ser/Thr and Man3(Fuc)GlcNAc2-*N*-Asn, respectively. However, the ability or inability of insect cells to synthesize and compartmentalize sialic acids and to produce sialylated glycans remains controversial. This is an important issue because terminal sialic acid residues play diverse biological roles in many glycoconjugates. While most work indicates that insect cell-derived glycoproteins are not sialylated, some well-controlled studies suggest that sialylation can occur. In evaluating this work, it is important to recognize that oligosaccharide structural determination is tedious work, due to the infinite diversity of this class of compounds. Furthermore, there is no universal method of glycan analysis; rather, various strategies and techniques can be used, which provide glycobiologists with relatively more or less precise and reliable results. Therefore, it is important to consider the methodology used to assess glycan structures when evaluating these studies. The purpose of this review is to survey the studies that have contributed to our current view of glycoprotein sialylation in insect cell systems, according to the methods used. Possible reasons for the disagreement on this topic in the literature, which include the diverse origins of biological material and experimental artifacts, will be discussed. In the final analysis, it appears that if insect cells have the genetic potential to perform sialylation of glycoproteins, this is a highly specialized function that probably occurs rarely. Thus, the production of sialylated recombinant glycoproteins in the baculovirus-insect cell system will require metabolic engineering efforts to extend the native protein glycosylation pathways of insect cells.

Key words: Baculovirus / Glycoproteins / Insect cells / Recombinant glycoproteins / Sialylation.

Introduction

Considering the rapid progress in sequencing the genomes of many organisms, including humans, the availability of expression systems that can be used to produce authentic mammalian proteins and glycoproteins has become crucial. One of the most important features of such a system is its glycosylation potential. Indeed, in order to study the biological properties of a recombinant glycoprotein or use it as a therapeutic agent, its glycosylation pattern must closely resemble the *in vivo* glycosylation pattern of the native product. Insect cells are widely used to produce recombinant proteins, as they can synthesize large quantities of a protein of interest when infected with powerful baculovirus-based gene expression vectors, and they can provide post-translational modifications similar to those provided by mammalian cells. Studies have shown that glycoproteins produced by all insect systems studied to date have O- and N-glycans with core structures similar or identical to those produced by all eukaryotes. In contrast, most studies suggest that insect cell-derived glycoproteins typically fail to acquire antennae of the N-acetyllactosaminyl type or peripheral sugars, especially sialic acids, which are commonly found on native mammalian glycans. Sialic acids play extremely important roles in glycoprotein biology. Because they are typically found as terminal residues on cell-surface glycoconjugates or circulatory components, they are involved in many cell–cell interactions, immunological reactions and in the clearance of circulating glycoproteins. Accordingly, the pres-

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[a] André Verbert died on 20 May 2000. We will miss his friendship and his enthusiasm. This review is dedicated to his memory.

ence or absence of sialic acids in insect cells is a significant and somewhat controversial issue regarding the use of these cells as recombinant glycoprotein factories.

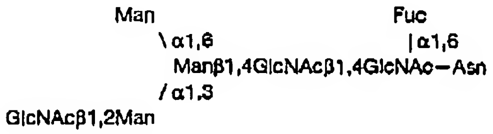
In addition to its biotechnological significance, the presence or absence of sialic acids in insects is fundamentally interesting from a phylogenic point of view. According to an early review (Warren, 1963), sialic acids had not been detected among the Coelenterata, Annelida or Sipunculoidea. In contrast, they had been found in all vertebrate species, cephalocords, and echinoderms and were found sporadically among some platyhelminths, molluscs, and arthropods. In some phyla, KDN (keto-deoxyneuraminic acid) was found in place of sialic acid. Among the arthropods, lobsters reportedly had sialic acids but, at that time, no sialic acids had been detected in any insect or arachnid species examined.

In this review, we will survey the studies that have contributed to our current view of the presence or absence of sialic acids in endogenous, viral and recombinant glycoproteins synthesized by insect cells, taking into account the methods used to determine glycan structures.

Current Knowledge of Insect Cell Protein Glycosylation

There is evidence that many of the glycoprotein processing events known to occur in mammalian cells also occur in insect cells. For a general review, see März *et al.* (1995).

The insect N-glycosylation pathway parallels the mammalian pathway up until formation of the following structure:



Man Fuc
\ α1,6 | α1,6
Manβ1,4GlcNAcβ1,4GlcNAc—Asn
/ α1,3
GlcNAcβ1,2Man

The major insect cell processing pathway downstream of this intermediate involves removal of the GlcNAc residue by a Golgi-associated N-acetylglucosaminidase, which produces a paucimannose type N-glycan (Altmann *et al.*, 1995; Wagner *et al.*, 1996a; Marchal *et al.*, 1999). This end-product is the most highly processed glycan found on most insect cell-derived N-linked glycoproteins. However, this does not appear to be the only N-glycan processing pathway found in insect cells, as various reports indicate that these cells also can produce subpopulations of some glycoproteins containing terminal N-acetylglucosaminyl, galactosyl, and sialyl residues. The O-glycosylation pathway in many lepidopteran insect cell lines produces glycoproteins containing GalNAcα-O-Ser/Thr and a subpopulation of these structures is further processed to produce the Galβ1,3GalNAcα-O-Ser/Thr core-1 structure (März *et al.*, 1995). Both of these O-glycans are potential acceptors for sialyl residues.

Requirements for Glycoprotein Sialylation

The occurrence of sialic acid in a glycoconjugate from a particular cell type implies that that cell can perform the following reaction:

non-sialylated acceptor + CMP-sialic acid → sialylated acceptor + CMP

where CMP is cytidine monophosphate.

Thus, the requirements for sialylation of a glycoconjugate are:

The acceptor: acceptors include N-glycoproteins, O-glycoproteins, and glycolipids, and the sialyl residue can be transferred to galactosyl, N-acetylgalactosaminyl or sialyl residues.

The enzyme: the sialyltransferases are a large family of enzymes, which all use CMP-Neu5Ac (CMP-N-acetylneuraminic acid) or its derivatives *e. g.* CMP-Neu5Gc (CMP-N-glycolylneuraminic acid) as a donor. These enzymes have high specificity toward the acceptor and generally recognize not only the monosaccharide to which the sialic acid is transferred, but a more complex glycan motif. For a review, see Harduin-Lepers *et al.* (1995).

The donor substrate: the sialylation reaction requires the activated form of sialic acid, CMP-Neu5Ac, or its derivatives, as mentioned above. The CMP-NeuAc synthase, in contrast to other sugar-nucleotide synthases, is a nuclear resident as shown by biochemical studies (for a review see Kean, 1991) and, more recently, *via* expression of the cloned murine cDNA (Münster *et al.*, 1998).

Proper subcellular localization of both enzyme and substrates: sialylation reactions occur in the *trans*-Golgi. Translocation of donor substrates into the lumen of the Golgi apparatus requires a specific CMP-Neu5Ac/CMP antiporter, which is well characterized in mammals (Eckhardt *et al.*, 1996, Hirschberg *et al.*, 1998).

How to Assess the Sialylation Ability of a Cell

One way to assess the ability of a tissue to sialylate glycoproteins is to measure sialyltransferase activity in that tissue using either endogenous or exogenous acceptors. These assays can be performed by incubating cell extracts, subcellular fractions, or tissue slices with radiolabeled CMP-Neu5Ac. A similar approach is metabolic labelling with radioactive mannosamine or Neu5Ac, although the way by which the latter, anionic precursor, is taken up by cells is not understood.

A totally different approach is to analyze endogenous or recombinant glycoproteins for the presence of sialyl residues. Some methods utilize neuraminidases, which can specifically remove sialic acids, whereas other methods utilize lectins, which can specifically bind sialic acids. Among the lectins, MAA (*Maackia amurensis* agglutinin) and SNA (*Sambucus nigra* agglutinin) are commonly used to detect α2,3 and α2,6 sialic acids, respectively. The use of neuraminidase must be coupled with a subsequent analysis of the biological or biophysical properties of the putatively sialylated molecule; for example, one might compare the electrophoretic mobilities of a glycoprotein before and after neuraminidase treatment. Lectins can be used to stain sialylated glycoproteins after electrophoresis and transfer to a membrane or they can be used for agglutination assays. Lectin-based assays must always be coupled with extensive controls, including endoglycosidase treatments, neuraminidase treatments, and/or the use of competing monosaccharides, to verify the carbohydrate specificity of the lectin binding results. More direct physico-chemical methods also can be used to detect sialic acids. While early work involved histochemical and colorimetric methods, more recent studies have involved the separation of glycan moieties by HPLC (high performance liquid chromatography), HPAEC (high pH anion exchange chromatography), or FACE (fluorophore-assisted carbohydrate electrophoresis), followed by structural determinations using mass spectrometry or NMR (nuclear magnetic resonance).

One factor that can complicate the measurement of sialyltransferase activities or sialic acids is degradation by sialidases, which have been detected in insect cells (Licari *et al.*, 1993), and could lead to false-negative results. The opposite problem is contamination with sialic acids or sialoglycoproteins derived from the animal serum used in the cell growth medium, which can produce false-positive results.

Sialylation of Endogenous Insect Cell Glycoproteins

Endogenous glycoproteins are defined as those that are encoded by the cellular genome, thus excluding any glycoprotein expressed during viral infection, including recombinant glycoproteins.

Detection of Sialyl Residues

In early studies, Vadgama and Kamat (1969) and Kamat (1971) used alcian blue staining, combined with mild acid hydrolysis and neuraminidase digestion to show the presence of sialic acids in the salivary glands of insects from several different orders. These conclusions were inconsistent with the results of Warren (1963), who observed no sialic acids in insects using the thiobarbituric acid method. Gee (1975) also suggested that the diuretic hormone of the tsetse fly *Glossina austeni* contained sialic acid because it lost its biological activity after neuraminidase treatment. A more recent study demonstrated that specific adhesion of *Plasmodium* ookinetes to the midgut epithelium of *Aedes aegypti* involves a midgut carbohydrate ligand and that free N-acetylneuraminic acid competes with the ookinetes in binding assays (Zieler *et al.*, 1999). However, these authors were unable to detect any sialic acids in mosquito midguts using a highly sensitive, HPLC-based fluorometric assay. Scolexin, a bacterially-induced immune protein specifically found in *Manduca sexta* larvae, has been shown to contain N-acetylneuraminic acid using GC-MS (gas chromatography-mass spectrometry) analysis (Kyriakides *et al.*, 1995).

Neu5Ac-specific lectins have been widely used to examine insect cell proteins for sialic acids, but have provided conflicting results. In one study, Davis and Wood (1995) used MAA and SNA blotting to identify sialylated glycoproteins in established insect cell lines from *Spodoptera frugiperda* (Sf21) and *Trichoplusia ni* (TN-368 and BTI-Tn-5B1-4). However, no proper specificity controls were included in their experiments. In another study, McCarthy and Fletcher (1992) were unable to detect sialic acids in seven different lepidopteran insect cell lines, including Sf21 and TN-368, using LPA (*Limulus polyphemus* agglutinin) agglutination assays. Lopez *et al.* (1999) also found no evidence of sialic acids using SNA and PNA (peanut agglutinin) lectin blotting analysis of endogenous glycoproteins from *Spodoptera frugiperda*, *Trichoplusia ni* and *Mamestra brassicae* and, in this study, the lectin blotting results were confirmed by MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry).

Finally, a precise two-dimensional HPLC and exoglycosidase analysis of membrane glycoprotein glycans from three different lepidopteran insect cell lines (Sf21, IZD-Mb-0503 and Bm-N) revealed no evidence of sialylation (Kubelka *et al.*, 1994).

In summary, the results of most, but not all, of these studies suggest that endogenous insect glycoproteins lack sialic acids. The inconsistency in these results might reflect cell type- and/or developmental stage-specific sialylation events. Indeed, sialic acids have been detected in *Drosophila melanogaster* by cytochemistry using LFA (*Limax flavus* agglutinin) and by a combination of gas-liquid chromatography and electron-impact mass spectrometry (Roth *et al.*, 1992). These authors also used blotting with an anti-polysialic acid antibody to demonstrate that homopolymers of α2,8 linked sialic acids were expressed only in embryos between 14 and 18 hours of age. These results show that sialic acids do occur in Diptera, and that some forms, *e. g.* polysialic acid, occur in a developmental stage-specific fashion, as shown previously for polysialylation of the mammalian neuronal adhesion molecule N-CAM (Vimr *et al.*, 1984, Seki and Arai, 1993).

Occurrence of Endogenous Sialyltransferase Activity

Some investigators have used *in vitro* assays with radiolabeled or fluorescent CMP-Neu5Ac as the donor substrate to examine insect cell lysates or subcellular fractions for sialyltransferase activities (Butters *et al.*, 1981, Hooker *et al.*, 1999 and Lopez *et al.*, 1999). No significant sialyltransferase activity has ever been detected in these assays, irrespective of the acceptor substrate used. In contrast, Hiruma and Riddiford (1988) reported that the granular phenoloxidase of the tobacco homworm, *Manduca saxta* was [^{14}C]-labeled when tissue slices were incubated with [^{14}C]Neu5Ac.

The difference in these results might reflect insect type- or tissue-specific differences in sialyltransferase activities, differences in established cell lines *versus* intact insects, or protein-specific differences, as the latter study focused on incorporation of radiolabeled sialic acid into a single insect glycoprotein.

Availability of Endogenous CMP-Neu5Ac

Few studies have examined the occurrence of the sialic acid donor, CMP-Neu5Ac, in insect systems. However, Hooker *et al.* (1999) recently reported that they were unable to detect any CMP-Neu5Ac by anion exchange chromatography of the soluble nucleotides extracted from uninfected Sf9, Sf21, or Ea4 cells or from baculovirus-infected Sf9 cells.

Glycoprotein Sialylation during Viral Infection

The granulosis viruses and the nucleopolyhedroviruses, both of which belong to the family Baculoviridae, can infect numerous insect species and are widely studied because of their potential applications both as biological insecticides and as eukaryotic gene expression vectors. The viruses classified within the genus baculovirus all consist of a nucleocapsid surrounded by an envelope, which includes virus-encoded glycoproteins that are translated and modified by the host cell. These glycoproteins can be used conveniently to study insect cellular protein glycosylation machinery because they are usually synthesized in large quantities. Using the lectin LPA, which binds to sialic acids, Russell and Consigli (1985) detected one sialylated glycoprotein synthesized during infection of *Trichoplusia ni* (TN-368) cells with *Plodia interpunctella* granulosis virus. In contrast, several studies of nucleopolyhedroviruses led to the conclusion that no viral glycoproteins were sialylated. Kretzschmar *et al.* (1994) demonstrated that neuraminidase treatment had no effect on the HPLC profile of the N-glycans isolated from baculovirus (*Autographa californica* nuclear polyhedrosis virus; AcMNPV)-infected Sf9 cells. They concluded that viral infection does not alter N-glycosylation and that Sf9 cells are not capable of sialylating glycoproteins. Jarvis and Finn (1995) used lectin blotting analysis to examine N-glycosylation of the major AcMNPV envelope glycoprotein, gp64 and found no evidence of sialic acid when gp64 was expressed in three different lepidopteran insect cell lines. However, gp64 was sialylated when it was expressed in mammalian cells. More recently, Jarvis *et al.* (1998b) constructed several gp64 mutants, each of which had only one of the five potential N-glycosylation sites, and found no evidence of sialic acid in any of the individual N-linked glycans by SNA lectin blotting. Finally, as part of their study, Hooker *et al.* (1999) showed that neither sialyltransferase activity nor CMP-Neu5Ac donor substrate was detectable in baculovirus-infected Sf9 cells, as mentioned above.

In addition to these insect-specific viruses, some animal viruses can grow in a wide variety of cultured cells, including cells of either mammalian or insect origin. This allows for a well-controlled comparison of protein glycosylation by both cell types, as glycosylation of virion proteins is mediated by cellular glycosylation pathways. The sialic acid content of isolated virions can be assayed by simple colorimetric methods. Schloemer and Wagner (1975) showed that sialylation of vesicular stomatitis virus grown in mosquito cells (*Aedes albopictus*) was very low, resulting in markedly reduced hemagglutinating activity. *In vitro* sialylation restored the hemagglutination titre of this virus to levels approaching those of the same virus grown in BHK-21 cells. Impaired sialylation has also been observed for the Semliki forest virus E2 glycoprotein produced by mosquito cells (Stollar, 1980) and purified Sindbis virus grown in mosquito cells, relative to the same virus grown in BHK cells (Stollar *et al.*, 1976; Hirsch *et al.*, 1981).

Thus, these results provide no evidence for significant induction of glycoprotein sialylation pathways during infection of insect cells by a variety of different viruses.

Sialylation of Recombinant Glycoproteins in Baculovirus-Insect Cell Expression Systems

Recombinant baculoviruses are commonly used as vectors for the high level expression of foreign genes under the transcriptional control of the viral polyhedrin or p10 promoters, which are exceptionally strong promoters [for reviews, see Miller (1988), Luckow and Summers (1988), Jarvis (1997), and Altmann *et al.* (1999)]. Examination of baculovirus-expressed glycoproteins for sialylation has provided conflicting results, which we will discuss according to the methods used for sialic acid detection and characterization.

Use of Lectins to Determine Glycan Structures

Vandenbroeck *et al.* (1994) showed that porcine interferon-γ produced by baculovirus-infected Sf9 cells could be stained with SNA and MAA, indicating that this recombinant protein had acquired α2,3 and α2,6-linked sialic acids. Similarly, Davis and Wood (1995) reported that human placental secreted alkaline phosphatase (SEAP) produced in Sf21 and TN-368 cell lines reacted with SNA and concluded that this protein had acquired α2,6-linked sialic acid. This conclusion was weakened by the lack of suitable controls, as discussed above. Similarly, the conclusion that a bovine leukemia virus glycoprotein expressed by recombinant baculovirus-infected insect cells contains sialic acid was weakened by the fact that it was based on the use of WGA (wheat germ agglutinin), which interacts poorly with sialyl residues and has other specificities (Russo *et al.*, 1998). Moreover, the culture medium used in this study contained 5% fetal calf serum, which could be a source of contaminating sialoglycoproteins, as discussed above. Finally, MAA competed with antibodies in a competitive ELISA test on the porcine lutropin receptor ectodomain expressed in Sf9 cells, indicating that this recombinant protein contained α2,3 sialic acid (Pajot-Augy *et al.*, 1999).

In contrast to these positive results, many investigators have been unable to detect sialic acid residues using lectin-based analyses of insect cell-derived recombinant glycoproteins. Among them, Lehmann *et al.* (1993) used Con A (concanavalin A) affinity chromatography to separate the N-glycans from a secreted, chimeric form of a human parainfluenza virus type 3 glycoprotein produced in Sf9 cells and their results revealed only oligomannosidic- and paucimannosidic glycans. Sialic acids were not found on human interferon ω1 expressed in Sf9 cells, as the only positive signal in lectin blots was observed with GNA (*Galanthus nivalis* agglutinin), which recognizes terminal mannose residues (Voss *et al.*, 1993). Sugyiama *et al.* (1993) used lectin blotting with DSA (*Datura stramonium* agglutinin), SNA, MAA and WGA to characterize O-glycosylation of human interferon-α2 synthesized by Sf9 cells and found no evidence of sialylation. Grossmann *et al.* (1997) expressed the human thyroid stimulating hormone (TSH) in Sf9, Sf21, and BTI-TN-5B1-4 (also known as High Five) cells and detected no terminally sialylated complex oligosaccharides using ConA and LFA. Finally, the WGA lectin blotting results of Van de Wiel *et al.* (1998) suggested that the bovine follicular stimulating hormone (FSH) does not acquire terminal sialic acid when expressed in Sf21 cells.

Use of Physico-Chemical Criteria

Electrophoretic Mobility of Glycoproteins When human melanotransferrin was expressed in baculovirus-infected or stably transfected lepidopteran insect cells (Sf9), neither form of the protein included the higher acid form seen with the native protein produced by human cells. This result was taken by Hegedus *et al.* (1999) as an indication that neither recombinant form of the protein was sialylated by insect cells. The lack of a change in electrophoretic mobility after neuraminidase treatment has also been used to demonstrate the absence of sialic acid in recombinant human complement subcomponent C1s (Luo *et al.*, 1992) and human α1-microglobulin (Wester *et al.*, 1997), both expressed in Sf9 cells.

Chromatographic Behaviour of Glycans The structures of the O-linked oligosaccharides from a baculovirus-expressed, truncated pseudorabies virus glycoprotein (gp50) were examined by Thomsen *et al.* (1990) using gel filtration and paper chromatography after metabolic labeling. The major carbohydrate structures found in this study were GalNAc and, to a lesser extent, Galβ1,3GalNAc, but both structures were devoid of sialic acid.

Hsu *et al.* (1997) used two-dimensional HPLC mapping to analyze the N-glycans on IgG produced by baculovirus-infected BTI-TN-5B1-4 (High Five) cells. The results of this study showed that *Trichoplusia ni* cells could produce biantennary N-glycans terminating with galactosyl residues; however, there was no evidence of sialylation.

Expression of recombinant human plasminogen has been studied in several insect cell lines, including *Spodoptera frugiperda* IPLB-Sf21AE (Davidson *et al.*, 1990 and Davidson and Castellino, 1991a), *Mamestra brassicae* IZD-MbO503 (Davidson and Castellino, 1991b), and *Manduca sexta* CM-1 (Davidson and Castellino, 1991b). These investigators used HPAEC analyses to examine the structures of the N-glycans on the recombinant plasminogen produced by these cells. The results revealed that the protein contained significant proportions of sialylated, complex N-linked oligosaccharides. The same results were obtained whether the cells were cultured in serum-containing or serum-free media. To date, this remains the only example in which high levels of sialylated complex oligosaccharides were observed by direct structural analyses of the glycans from an insect cell-derived glycoprotein. Synthesis of these complex-type oligosaccharides was shown to be dependent on the elapsed post-infection time (Davidson and Castellino, 1991a). It has been suggested that human plasminogen might be a particularly good substrate for limited amounts of terminal processing enzymes and/or a particularly poor substrate for the processing N-acetylglucosaminidase found in these cells. These observations imply that insect cells have the glycosyltransferase genes required for the assembly of complex N-linked oligosaccharides and that these genes can be expressed during infection. However, using this same method of glycan analysis (HPAEC), Butters *et al.*, (1998) found no evidence of sialylated species among the N-glycans from HIVgp120 expressed in Sf9 cells.

Glycan Analysis by Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) Sialylation of secreted alkaline phosphatase (SeAP) produced in *Trichoplusia ni* larvae, which had been detected by lectin blotting (Davis and Wood, 1995), was reinvestigated by this same group (Kulakosky *et al.*, 1998) using the FACE method. This analysis failed to confirm the presence of sialyl residues on SeAP, irrespective of the host used for production, which included a variety of established cell lines (*Lymantria dispar*, *Heliotis virescens*, *Bombyx mori*) and larvae (*Spodoptera frugiperda*, *Trichoplusia ni*, *H. virescens*, *B. mori* and *Danaus plaxippus*). According to the authors, the discrepancy between their FACE and lectin blotting results could have resulted either from coimmunoprecipitation of a contaminating sialylated glycoprotein of the same molecular mass or from non-specific binding in their lectin blots. By adding mannosamine, which can increase intracellular sugar nucleotide pools, Donaldson *et al.* (1999) demonstrated that N-glycosylation of SeAP in

Sf21 cells was extended to include terminal N-acetylglucosamine, but neither galactosylated nor sialylated structures were detected.

Glycan Analysis by Mass Spectrometry Mass spectrometry, which is one of the most powerful tools available for carbohydrate structural determinations, has been used since 1993 to characterize *O*- and *N*-linked glycans on insect cell-derived recombinant glycoproteins. Using plasma desorption mass spectrometry Sugyiama *et al.* (1993) found no evidence of sialylated O-glycans on human interferon α2 produced in Sf9 cells. Fast atom bombardment mass spectrometry of glycopeptides allowed Grabenhorst *et al.* (1993) to demonstrate that human interleukin-2 from baculovirus-infected Sf21 cells contained exclusively fucosylated paucimannose type N-glycans. The N-glycans on interferon γ produced by two insect cell lines (Ea4 and Sf9) were studied by matrix-assisted laser desorption/time of flight (MALDI-TOF) mass spectrometry (Ogonah *et al.*, 1996). Whereas the recombinant protein produced by Sf9 cells had only paucimannose-type glycans (see also Hooker *et al.*, 1999), the same protein produced by Ea4 cells contained N-glycans with terminal GlcNAc and Gal residues. However, even the Ea4 cells failed to produce N-glycans containing sialic acid. This same method was used to demonstrate that human lactoferrin produced by baculovirus-infected Sf9 cells had only non-sialylated, truncated N-glycans (Salmon *et al.*, 1997). MALDI and electrospray mass spectrometry analyses allowed Lopez *et al.* (1997) to elucidate the structures of the N-glycans on baculovirus-expressed bovine lactoferrin produced in *Mamestra brassicae* cells. Two families of oligosaccharides were found: one consisted of oligomannosidic type glycans (Man_8 to $Man_6GlcNAc_2$) and the other consisted of short, partially fucosylated paucimannose-type glycans, but none were sialylated. Most recently, Rudd *et al.* (2000) used MALDI-TOF to demonstrate the presence of a subpopulation of terminally galactosylated N-glycans on the third eight-cysteine domain of the latent transforming growth factor-β binding protein-1 expressed in baculovirus-infected High Five cells. However, they detected no sialylated structures.

Engineering Glycoprotein Processing Pathways in the Baculovirus-Insect Cell Expression System

Considering that the vast majority of N-glycans on glycoproteins synthesized by insect cells are of the oligomannosidic or paucimannosidic type, the relative inability of these cells to produce sialylated glycoproteins could reflect the absence of functional levels of terminal glycosyltransferases in these cells. This idea led some investigators to attempt to modify the protein N-glycosylation pathway in baculovirus-infected insect cells by introducing exogenous glycosyltransferase activities into the system (for a general review, see Jarvis *et al.*, 1998a).

Wagner *et al.* (1996b) demonstrated that the N-glycans of fowl-plague hemagglutinin expressed in Sf9 cells could be elongated by coexpression of human β1,2-N-acetylglucosaminyltransferase I (GNT-I). These authors used two different recombinant baculoviruses, one expressing the hemagglutinin and the other expressing the human GNT-I gene, to coinfect Sf9 cells. Glycosylation of hemagglutinin was evaluated by a terminal galactosylation assay and HPAEC analyses. The results indicated that human GNT-I coexpression resulted in the production of hemagglutinin molecules with a four-fold higher content of N-glycans containing terminal GlcNAc residues. However, completely galactosylated and sialylated complex-type oligosaccharide side chains were not observed. In another study, Jarvis and Finn (1996) used a new type of baculovirus vector that can express foreign genes immediately after infection under the control of the promoter from the baculoviral *ie1* gene to express bovine β1,4-galactosyltransferase. This resulted in galactosylation of the viral glycoprotein, gp64, as demonstrated by RCA (*Ricinus communis* agglutinin) lectin blotting with appropriate controls. The same results were obtained when Sf9 cells were stably transformed to constitutively express bovine β1,4-galactosyltransferase, then infected with wild type AcMNPV (Hollister *et al.*, 1998). Infection of these stably-transformed cells (Sfβ4GalT) with a conventional recombinant baculovirus expression vector encoding human plasminogen activator also resulted in galactosylation of the recombinant protein, as evidenced by RCA blotting. However, upregulation of galactosyltransferase activity failed to produce any glycoproteins that bound to SNA, indicating that this did not induce production of α2,6-sialylated glycoproteins. In their review article, Jarvis *et al.* (1998a) reported that infection of Sfβ4GalT cells with an immediate early baculovirus vector encoding a mammalian α2,6-sialyltransferase under the transcriptional control of the *ie1* promoter resulted in production of gp64 that bound specifically to both RCA and SNA. These findings (Hollister and Jarvis, 2001) suggest that up-regulation of both transferase activities allows Sf9 cells to produce a foreign glycoprotein with N-linked glycans containing both β-linked galactose and terminal α2,6-linked sialic acid. It is important to note that this conclusion implies that Sf9 cells can produce and transport CMP-sialic acid, which is required for glycoprotein sialylation in addition to the sialyltransferase activity. This implication is not supported by the results of Hooker *et al.* (1999), who were unable to detect CMP-sialic acid in uninfected or infected Sf9 cells, as discussed above. Based on this finding, the latter group concluded that genetic modification of N-glycan processing in Sf9 cells will be constrained to terminal galactosylation. Hopefully, future studies will clarify the discrepancy between these two different viewpoints.

Conclusion

Compared to polypeptides and nucleic acids, primary structural analysis of glycans is far more complicated due to the huge diversity of linkages between monosaccharides.

Methods of glycan structural analysis include colorimetry, endo- and exoglycosidase digestions, permethylation and analysis of methylated monosaccharides by gas-liquid chromatography, the use of lectins with well-defined carbohydrate-binding specificities, and various chromatographic methods, particularly HPLC and HPAEC. However, the application of NMR and mass spectrometry methods has provided a technical leap in glycan structural analysis. These latter methods have been used to establish that insect cells can modify proteins by both O- and N-glycosylation. In addition, these methods have established that the major processed O- and N-glycan species found on endogenous and most baculovirus-expressed recombinant glycoproteins are (Galß1,3)GalNAc-O-Ser/Thr and Man3(Fuc)GlcNAc2-*N*-Asn, respectively. Among the results obtained using other technical approaches, some suggest that insects can produce sialylated glycoproteins, whereas most suggest that they cannot. There are many potential reasons for the inconsistency in these results, including differences in the sources and nature of materials used for analysis. Some studies have focused on insect cell lines, others on insects. Many different cell lines, derived from different insects, different tissues, and cultured under different conditions have been used for these studies. Some studies have examined glycans from total glycoprotein fractions, some from total membrane glycoprotein fractions, and most have focused on a single, purified model glycoprotein. Among the purified model proteins, some studies have examined endogenous proteins, while others have examined recombinant proteins. Other potential reasons for the different conclusions from different studies could include experimental artifacts, such as the loss of sialic acids, the introduction of sialic acids or sialoglycoproteins, or the absence of proper experimental controls, as discussed above. Finally, it is imperative to recognize that it is extremely difficult to generalize about protein glycosylation pathways in 'insect cells', as insects are an incredibly diverse group of animals. Nonetheless, even with all these caveats, we may still conclude that the production and compartmentalization of sialic acids and glycoprotein sialylation are not major biochemical processes in insect cells. To explain the exceptional observations of sialic acids or sialoglycoproteins in insects, we can speculate that these processes might be highly specialized or might occur only in a tissue- or developmental stage-specific fashion. Similarly, we can speculate that these processes might occur at only nominal, usually undetectable levels in established insect cell lines and, on rare occasions, these low level processes can yield sialylated glycoproteins. The overall conclusion that insect cells generally do not produce sialylated glycoproteins, with rare exceptions among specialized cells or individual model glycoproteins, is consistent with the results of all well-analyzed studies in the current literature.

Based on these conclusions, the development of engineered insect cells able to perform mammalian-type glycosylation can still be considered as a promising field. One can expect that the introduction of mammalian genes encoding not only glycosyltransferases but also the enzymes responsible for sugar-nucleotides (especially CMP-sialic acid, as the endogenous pool must be very reduced) biosynthesis and transport can significantly modify the glycosylation pattern of insect cell-derived glycoproteins. However, since insects seem to possess the genetic potential to perform complex-type glycosylation, which will hopefully be confirmed by future genetic studies, another direction for metabolic engineering of insect cell glycosylation will be to manipulate the expression levels of endogenous glycoprotein-processing enzymes. These new developments will require basic molecular studies to understand the factors that govern the precise regulation of the genes involved in complex-type glycan synthesis.

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References

Altmann, F., Schwihla, H., Staudacher, E., Glossl, J. and März, L. (1995). Insect cells contain an unusual, membrane-bound beta-N-acetylglucosaminidase probably involved in the processing of protein N-glycans. J. Biol. Chem. *270*, 17344–17349.

Altmann, F., Staudacher, E., Wilson, I.B.H. and März, L. (1999). Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj. J. *16*, 109–123.

Butters, T.D., Hughes, R.C. and Vischer, P. (1981). Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. Biochim. Biophys. Acta *640*, 672–686.

Butters, T.D., Yudkin, B., Jacob, G.S. and Jones, I.M. (1998). Structural characterization of the N-linked oligosaccharides derived from HIVgp120 expressed in lepidopteran cells. Glycoconj. J. *15*, 83–88.

Davidson, D.J., Fraser, M.J. and Castellino, F.J. (1990). Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (*Spodoptera frugiperda*) cells. Biochemistry *29*, 5584–5590.

Davidson, D.J. and Castellino, F.J. (1991a). Asparagine-linked oligosaccharide processing in lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. Biochemistry *30*, 6165–6174.

Davidson, D.J. and Castellino, F.J. (1991b). Structures of the asparagine-289-linked oligosaccharides assembled on recombinant human plasminogen expressed in a *Mamestra brassicae* cell line (IZD-MBO503). Biochemistry *30*, 6689–6696.

Davis, T.R. and Wood, H.A. (1995). Intrinsic glycosylation potentials of insect cell cultures and insect larvae. In Vitro Cell. Dev. Biol. Anim. *31*, 659–663.

Donaldson, M., Wood, H.A., Kulakosky, P.C. and Shuler, M.L. (1999). Use of mannosamine for inducing the addition of outer arm N- acetylglucosamine onto N-linked oligosaccharides of recombinant proteins in insect cells. Biotechnol. Prog. *15*, 168–173.

Eckhardt, M., Muehlenhoff, M., Bethe, A., and Gerardy-Schahn, R. (1996) Exression cloning of the Golgi CMP-sialic acid transporter. Proc. Natl. Acad. Sci. USA *93*, 7572–7576.

Gee, J.D. (1975). The control of diuresis in the tsetse fly *Glossina austeni*: a preliminary investigation of the diuretic hormone. J. Exp. Biol. *63*, 391–401.

Grabenhorst, E., Hofer, B., Nimtz, M., Jager, V. and Conradt, H.S. (1993). Biosynthesis and secretion of human interleukin 2 glycoprotein variants from baculovirus-infected Sf21 cells. Characterization of polypeptides and posttranslational modifications. Eur. J. Biochem. *215*, 189–197.

Grossmann, M., Wong, R., Teh, N.G., Tropea, J.E., East-Palmer, J., Weintraub, B.D. and Szkudlinski, M.W. (1997). Expression of biologically active human thyrotropin (hTSH) in a baculovirus system: effect of insect cell glycosylation on hTSH activity *in vitro* and *in vivo*. Endocrinology *138*, 92–100.

Harduin-Lepers, A., Recchi, M.A. and Delannoy, P. (1995). 1994, the year of sialyltransferases. Glycobiology *5*, 741–758.

Hegedus, D.D., Pfeifer, T.A., Theilmann, D.A., Kennard, M.L., Gabathuler, R., Jefferies, W.A. and Grigliatti, T.A. (1999). Differences in the expression and localization of human melanotransferrin in lepidopteran and dipteran insect cell lines. Protein Expr. Purif. *15*, 296–307.

Hirsch, R.L., Griffin, D.E. and Winkelstein, J.A. (1981). Host modification of Sindbis virus sialic acid content influences alternative complement pathway activation and virus clearance. J. Immunol. *127*, 1740–1743.

Hirschberg, C.B., Robbins, P.W. and Abeijon, C. (1998). Transporters of nucleotide sugars, ATP, and nucleotide sulfate in the endoplasmic reticulum and Golgi apparatus. Annu. Rev. Biochem. *67*, 49–69.

Hiruma, K. and Riddiford, L.M. (1988). Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. Dev. Biol. *130*, 87–97.

Hollister, J.R., and Jarvis, D.L. (2001). Engineering Lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1,4-galactosyltransferase and α2,6-sialyltransferase genes. Glycobiology, in press.

Hollister, J.R., Shaper, J.H. and Jarvis, D.L. (1998). Stable expression of mammalian beta 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. Glycobiology *8*, 473–480.

Hooker, A.D., Green, N.H., Baines, A.J., Bull, A.T., Jenkins, N., Strange, P.G. and James, D.C. (1999). Constraints on the transport and glycosylation of recombinant IFN-gamma in Chinese hamster ovary and insect cells. Biotechnol. Bioeng. *63*, 559–572.

Hsu, T.A., Takahashi, N., Tsukamoto, Y., Kato, K., Shimada, I., Masuda, K., Whiteley, E.M., Fan, J.Q., Lee, Y.C. and Betenbaugh, M.J. (1997). Differential N-glycan patterns of secreted and intracellular IgG produced in *Trichoplusia ni* cells. J. Biol. Chem. *272*, 9062–9070.

Jarvis, D.L., (1997). Baculovirus expression vectors. In: The Baculoviruses, L.K. Miller, ed. (New York: Plenum Publishing Corporation) pp. 389–431.

Jarvis, D.L and Finn, E.E. (1996). Modifying the insect cell N-glycosylation pathway with immediate early baculovirus expression vectors. Nature Biotechnol. *14*, 1288–1292.

Jarvis, D.L. and Finn, E.E. (1995). Biochemical analysis of the N-glycosylation pathway in baculovirus- infected lepidopteran insect cells. Virology *212*, 500–511.

Jarvis, D.L., Kawar, Z.S. and Hollister, J.R. (1998a). Engineering N-glycosylation pathways in the baculovirus-insect cell system. Curr. Opin. Biotechnol. *9*, 528–533.

Jarvis, D.L., Wills, L., Burow, G. and Bohlmeyer, D.A. (1998b). Mutational analysis of the N-linked glycans on *Autographa californica* nucleopolyhedrovirus gp64. J. Virol. *72*, 9459–9469.

Kamat, D.N. (1971). The occurrence of sialic acids in invertebrates. Folia. Histochem. Cytochem. (Krakow) *9*, 189–192.

Kean, E.L. (1991). Sialic acid activation. Glycobiology *1*, 441–447.

Kretzschmar, E., Geyer, R. and Klenk, H.D. (1994). Baculovirus infection does not alter N-glycosylation in *Spodoptera frugiperda* cells. Biol. Chem. Hoppe-Seyler *375*, 23–27.

Kubelka, V., Altmann, F., Kornfeld, G. and März, L (1994). Structures of the N-linked oligosaccharides of the membrane glycoproteins from three lepidopteran cell lines (Sf-21, IZD-Mb-0503, Bm-N). Arch. Biochem. Biophys. *308*, 148–157.

Kulakosky, P.C., Hughes, P.R. and Wood, H.A. (1998). N-Linked glycosylation of a baculovirus-expressed recombinant glycoprotein in insect larvae and tissue culture cells. Glycobiology *8*, 741–745.

Kyriakides, T.R., McKillip, J.L. and Spence, K.D. (1995). Biochemical characterization, developmental expression, and induction of the immune protein scolexin from *Manduca sexta*. Arch. Insect Biochem. Physiol. *29*, 269–280.

Lehman, D.J., Roof, L.L., Brideau, R.J., Aeed, P.A., Thomsen, D.R., Elhammer, A.P., Wathen, M.W. and Homa, F.L. (1993). Comparison of soluble and secreted forms of human parainfluenza virus type 3 glycoproteins expressed from mammalian and insect cells as subunit vaccines. J. Gen. Virol. *74*, 459–469.

Licari, P.J., Jarvis, D.L. and Bailey, J.E. (1993). Insect cell hosts for baculovirus expression vectors contain endogenous exoglycosidase activity. Biotechnol. Prog. *9*, 146–152.

Lopez, M., Coddeville, B., Langridge, J., Plancke, Y., Sautiere, P., Chaabihi, H., Chirat, F., Harduin-Lepers, A., Cerutti, M., Verbert, A. and Delannoy, P. (1997). Microheterogeneity of the oligosaccharides carried by the recombinant bovine lactoferrin expressed in *Mamestra brassicae* cells. Glycobiology *7*, 635–651.

Lopez, M., Tetaert, D., Juliant, S., Gazon, M., Cerutti, M., Verbert, A. and Delannoy, P. (1999). O-glycosylation potential of lepidopteran insect cell lines. Biochim. Biophys. Acta *1427*, 49–61.

Luckow, L.A. and Summers, M.D. (1988). Trends in the development of baculovirus expression vectors. Bio/Technology *6*, 47–55.

Luo, C., Thielens, N.M., Gagnon, J., Gal, P., Sarvari, M., Tseng, Y., Tosi, M., Zavodszky, P., Arlaud, G.J. and Schumaker, V.N. (1992). Recombinant human complement subcomponent C1s lacking beta- hydroxyasparagine, sialic acid, and one of its two carbohydrate chains still reassembles with C1q and C1r to form a functional C1 complex. Biochemistry *31*, 4254–4262.

März, L., Altmann, F., Staudacher, E. and Kubelka, V. (1995). Protein glycosylation in insects. In: Glycoproteins, J. Montreuil, H. Schachter, and J.F.G. Vliegenthart, eds. (Amsterdam, The Netherlands: Elsevier) pp. 543–563.

Marchal, I., Mir, A.M., Kmiecik, D., Verbert, A. and Cacan, R. (1999). Use of inhibitors to characterize intermediates in the processing of N- glycans synthesized by insect cells: a metabolic study with Sf9 cell line. Glycobiology *9*, 645–654.

McCarthy, W.J. and Fletcher, K.A. (1992). Cell-surface glycoconjugate diversity among lepidopteran cell lines. In Vitro Cell. Dev. Biol. *28A*, 700–702.

Miller, L.K. (1988). Baculoviruses as gene expression vectors. Annu. Rev. Microbiol. *42*, 177–199.

Münster, A-K., Eckhardt, M., Potvin, B., Mühlenhoff, M., Stanley, P. and Gerardy-Schahn, R. (1998). Mammalian cytidine 5'-monophosphate N-acetylneuraminic acid synthetase: A nuclear protein with evolutionarily conserved motifs. Proc. Natl. Acad. Sci. USA *95*, 9140–9145.

Ogonah, O.W., Freedman, R.B., Jenkins, N., Patel, K. and Rooney, B.C. (1996). Isolation and characterization of an insect cell line able to perform complex N-linked glycosylation on recombinant glycoproteins. Bio/Technology *14*, 197–202.

Pajot-Augy, E., Bozon, V., Remy, J.J., Couture, L. and Salesse, R. (1999). Critical relationship between glycosylation of recombinant lutropin receptor ectodomain and its secretion from baculovirus-infected insect cells. Eur. J. Biochem. *260*, 635–648.

Roth, J., Kempf, A., Reuter, G., Schauer, R. and Gehring, W.J. (1992). Occurrence of sialic acids in *Drosophila melanogaster*. Science *256*, 673–675.

Rudd, P.M., Downing, A.K., Cadene, M., Harvey, D.J., Wormald, M.R., Weir, I., Dwek, R.A., Rifkin, D.B. and Gleizes, P.E. (2000). Hybrid and complex glycans are linked to the conserved N-glycosylation site of the third eight-cysteine domain of LTBP-1 in insect cells. Biochemistry *39*, 1596–1603.

Russell, D.L. and Consigli, R.A. (1985). Glycosylation of purified enveloped nucleocapsids of the granulosis virus infecting *Plodia interpunctella* as determined by lectin binding. Virus Res. *4*, 83–91.

Russo, S., Montermini, L., Berkovitz-Siman-Tov, R., Ponti, W. and Poli, G. (1998). Expression of bovine leukemia virus ENV glycoprotein in insect cells by recombinant baculovirus. FEBS Lett. *436*, 11–16.

Salmon, V., Legrand, D., Georges, B., Slomianny, M.C., Coddeville, B. and Spik, G. (1997). Characterization of human lactoferrin produced in the baculovirus expression system. Protein Expr. Purif. *9*, 203–210.

Schloemer, R.H. and Wagner, R.R. (1975). Mosquito cells infected with vesicular stomatitis virus yield unsialylated virions of low infectivity. J. Virol. *15*, 1029–1032.

Seki, T. and Arai, Y. (1993). Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci. Res. *17*, 265–290.

Stollar, V. (1980). Togaviruses in cultured arthropod cells. In: The togaviruses: Biology, Structure, Replication R.W. Schlesinger, ed. (New York, USA: Academic Press), pp. 584–622.

Stollar, V., Stollar, B.D., Koo, R., Harrap, K.A. and Schlesinger, R.W. (1976). Sialic acid contents of sindbis virus from vertebrate and mosquito cells. Equivalence of biological and immunological viral properties. Virology *69*, 104–115.

Sugyiama, K., Ahorn, H., Maurer-Fogy, I. and Voss, T. (1993). Expression of human interferon-alpha 2 in Sf9 cells. Characterization of O-linked glycosylation and protein heterogeneities. Eur. J. Biochem. *217*, 921–927.

Thomsen, D.R., Post, L.E. and Elhammer, A.P. (1990). Structure of O-glycosidically linked oligosaccharides synthesized by the insect cell line Sf9. J. Cell. Biochem. *43*, 67–79.

Vadgama, M.R. and Kamat, D.N. (1969). Sialic acids in the salivary glands of some insects with different feeding habits. Histochemie *19*, 184–188.

Van de Wiel, D.F., Van Rijn, P.A., Meloen, R.H. and Moormann, R.J. (1998). High-level expression of biologically active recombinant bovine follicle stimulating hormone in a baculovirus system. J. Mol. Endocrinol. *20*, 83–98.

Vandenbroeck, K., Willems, L., Billiau, A., Opdenakker, G. and Huybrechts, R. (1994). Glycoform heterogeneity of porcine interferon-gamma expressed in Sf9 cells. Lymphokine Cytokine Res. *13*, 253–258.

Vimr, E.R., McCoy, R.D., Vollger, H.F., Wilkison, N.C. and Troy, F.A. (1984). Use of prokaryotic-derived to identify poly(sialic acid) in neonatal neuronal membranes. Proc. Natl. Acad. Sci. USA *81*, 1971–1975.

Voss, T., Ergulen, E., Ahorn, H., Kubelka, V., Sugiyama, K., Maurer-Fogy, I. and Glossl, J. (1993). Expression of human interferon omega 1 in Sf9 cells. No evidence for complex-type N-linked glycosylation or sialylation. Eur. J. Biochem. *217*, 913–919.

Wagner, R., Geyer, H., Geyer, R. and Klenk, H.D. (1996a). N-acetyl-beta-glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. J. Virol. *70*, 4103–4109.

Wagner, R., Liedtke, S., Kretzschmar, E., Geyer, H., Geyer, R. and Klenk, H.D. (1996b). Elongation of the N-glycans of fowl plague virus hemagglutinin expressed in *Spodoptera frugiperda* (Sf9) cells by coexpression of human beta 1,2-N-acetylglucosaminyltransferase I. Glycobiology *6*, 165–175.

Warren, L. (1963). The distribution of sialic acids in nature. Comp. Biochem. Physiol. *10*, 153–171.

Wester, L., Johansson, M.U. and Akerstrom, B. (1997). Physicochemical and biochemical characterization of human alpha 1-microglobulin expressed in baculovirus-infected insect cells. Protein Expr. Purif. *11*, 95–103.

Zieler, H., Nawrocki, J.P. and Shahabuddin, M. (1999). *Plasmodium gallinaceum* ookinetes adhere specifically to the midgut epithelium of *Aedes aegypti* by interaction with a carbohydrate ligand. J. Exp. Biol. *202*, 485–495.

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